Liza M. Walsh
Marc D. Haefner
Christine I. Gannon
WALSH PIZZI O'REILLY FALANGA LLP
Three Gateway Center
100 Mulberry Street, 15th Floor
Newark, New Jersey 07102
(973) 757-1100

OF COUNSEL:

David T. Pritikin
Steven J. Horowitz
SIDLEY AUSTIN LLP
One South Dearborn
Chicago, Illinois 60603
(312) 853-7000

Vernon M. Winters
SIDLEY AUSTIN LLP
555 California Street
San Francisco, CA 94104
(415) 772-1200

ADDITIONAL COUNSEL LISTED
ON SIGNATURE PAGE

*Attorneys for Immunex Corporation and Amgen Manufacturing, Limited*

David E. De Lorenzi
Charles H. Chevalier
GIBBONS P.C.
One Gateway Center
Newark, NJ 07102-5310
(973) 596-4500

OF COUNSEL:

David I. Berl
Aaron Maurer
Thomas S. Fletcher
WILLIAMS & CONNOLLY LLP
725 Twelfth St. NW
Washington, D.C. 20005
(202) 434-5000

*Attorneys for Hoffmann-La Roche Inc.*

**IN THE UNITED STATES DISTRICT COURT**
**FOR THE DISTRICT OF NEW JERSEY**

| | |
|---|---|
| IMMUNEX CORPORATION, AMGEN MANUFACTURING, LIMITED, and HOFFMANN-LA ROCHE INC.<br><br>Plaintiffs<br><br>v.<br><br>SAMSUNG BIOEPIS CO., LTD.,<br><br>Defendant. | Civil Action No. 2:19-cv-11755-CCC-MF<br><br>**FIRST AMENDED COMPLAINT & DEMAND FOR A JURY TRIAL** |

# FIRST AMENDED COMPLAINT

Plaintiffs, Immunex Corporation, Amgen Manufacturing, Limited, and Hoffmann-La Roche Inc. (collectively, "Plaintiffs"), by and through their undersigned attorneys, for their First Amended Complaint ("the Complaint") against Defendant Samsung Bioepis Co., Ltd. ("Bioepis") allege as follows:

## I. THE PARTIES

### A. Plaintiffs

1. Immunex Corporation ("Immunex") is a corporation organized and existing under the laws of the State of Washington with its principal place of business at One Amgen Center Drive, Thousand Oaks, California 91320. Amgen Inc. acquired Immunex in July 2002, and Immunex became a wholly-owned subsidiary of Amgen Inc.

2. Amgen Manufacturing, Limited ("AML") is a corporation existing under the laws of the Territory of Bermuda, with its principal place of business at Road 31 km 24.6, Juncos, Puerto Rico 00777. AML is a wholly-owned subsidiary of Amgen Inc.

3. Hoffmann-La Roche Inc. ("Roche") is a corporation organized and existing under the laws of the State of New Jersey with its principal place of business at 150 Clove Road, Suite 8, Little Falls, New Jersey 07424.

### B. Bioepis

4. On information and belief, Bioepis is a corporation organized and existing under the laws of South Korea, with its principal place of business at 107, Cheomdan-daero Yeonsu-gu Incheon, 406-840 South Korea. On information and belief, Bioepis develops, manufactures, and seeks regulatory approval for biosimilar products, and imports, markets, distributes, offers to sell, and sells those biosimilar products in the State of New Jersey and throughout the United States.

## II. NATURE OF THE ACTION

5. This is an action for patent infringement arising under 35 U.S.C. § 271, including § 271(e)(2)(C)(ii), which was enacted in 2010 as part of the Biologics Price Competition and Innovation Act ("the BPCIA"), and for relief under the BPCIA. This action involves patents that cover etanercept (the active ingredient of the biologic drug product Enbrel®), its method of manufacture, and certain materials used in its manufacture. Immunex and AML (collectively, "Immunex/AML") and Roche bring this suit to enjoin Bioepis from infringing their patents and to secure any recoverable damages resulting from Bioepis's infringement.

6. The asserted patents (collectively, "the Patents-In-Suit") are as follows:

- U.S. Patent No. 8,063,182 ("the '182 Patent"),
- U.S. Patent No. 8,163,522 ("the '522 Patent") (collectively, the '182 and '522 Patents are the "Roche Patents"),
- U.S. Patent No. 6,872,549 ("the '549 Patent"),
- U.S. Patent No. 6,924,124 ("the '124 Patent"),
- U.S. Patent No. 7,157,557 ("the '557 Patent") (collectively, the '549, '124, and '557 Patents are the "Immunex Patents").

7. Roche owns the '182 and '522 Patents. Immunex is the exclusive licensee of all commercial rights in the Roche Patents, including all rights to sell Enbrel® in the United States and its territories.

8. Immunex owns the '549, '124, and '557 Patents.

9. Immunex has granted AML an exclusive license (or, with respect to the '182 and '522 Patents, an exclusive sublicense) to the Patents-In-Suit.

10. According to files available at https://www.accessdata.fda.gov/scripts/cder/daf/index.cfm?event=overview.process&ApplNo=7

61066, on April 25, 2019, the U.S. Food and Drug Administration ("FDA"), approved Bioepis's abbreviated Biologics License Application 761066 ("BLA"). On information and belief, Bioepis submitted that BLA pursuant to the BPCIA, specifically 42 U.S.C. § 262(k) (also known as § 351(k) of the Public Health Service Act ("PHSA")), seeking authorization from the FDA to engage in the commercial manufacture, use, or sale of a biosimilar version of Immunex's Enbrel®, which Bioepis calls Eticovo (etanercept-ykro).

11. The BPCIA created an abbreviated pathway for the approval of biosimilar versions of approved biologic drugs. Subject to certain conditions, the abbreviated pathway (also known as "the (k) pathway") permits a biosimilar applicant (here, Bioepis) to rely on the prior clinical tests, data, and results, and the prior licensure and approval status, of the innovative biological product (here, Enbrel®). Immunex is the sponsor of the reference product, Enbrel®, which the FDA has approved for a number of different indications (*i.e.*, therapeutic uses).

12. As alleged herein, Bioepis infringed the Patents-In-Suit under 35 U.S.C. § 271(e)(2)(C)(ii) when it submitted its BLA seeking FDA approval to engage in the commercial manufacture, use, or sale of Bioepis's etanercept biosimilar product before the expiration of the Patents-In-Suit. As described in § IV.D below, Bioepis did not engage in the information exchange provided under the BPCIA and failed to provide Immunex with its BLA or information described in 42 U.S.C. § 262(*l*)(2)(A). In view of Bioepis's withholding of its BLA and such information, Immunex hereby asserts infringement of patents that could be identified pursuant to 42 U.S.C. § 262(*l*)(3)(A)(i). *See* 35 U.S.C. § 271(e)(2)(C)(ii).

13. As alleged herein, Bioepis would also infringe one or more claims of the Roche Patents under 35 U.S.C. § 271(a) and/or (g) should it make, use, offer for sale, or sell within the

United States, or import into the United States Bioepis's etanercept biosimilar product before the expiration of the Roche Patents.

## III. JURISDICTION AND VENUE

### A. Subject-Matter Jurisdiction

14. This Court has subject-matter jurisdiction over Immunex/AML and Roche's claims under 28 U.S.C. §§ 1331, 1338(a), 2201(a), and 2202.

### B. Personal Jurisdiction

15. This Court has personal jurisdiction over Bioepis by virtue of the fact that, on information and belief, Bioepis filed a BLA seeking approval from the FDA to engage in the commercial manufacture, use or sale of Bioepis's biosimilar product in the State of New Jersey and throughout the United States, which directly gives rise to Plaintiffs' claims of patent infringement. On information and belief, the FDA approved that application on April 25, 2019.

16. On information and belief, Bioepis, by itself or through others, intends to use, induce others to use, offer for sale, sell within the United States, and import into the United States, including the District of New Jersey, its etanercept biosimilar product.

17. This Court also has personal jurisdiction over Bioepis by virtue of Bioepis's contacts with New Jersey and the exercise of such personal jurisdiction is fair and reasonable. Litigating this suit in New Jersey does not burden Bioepis. For example, Bioepis did not object to personal jurisdiction when sued by another patent holder in this district. *Janssen Biotech, Inc. v. Samsung Bioepis, Co. Ltd*., Case No. 2:17-cv-03524 (MCA).

### C. Venue

18. Venue is proper in this District pursuant to 28 U.S.C. § 1391(c)(3). Bioepis is a foreign corporation and is therefore subject to suit in any judicial district. *Brunette Machine*

*Works, Ltd. v. Kockum Industries, Inc*., 406 U.S. 706, 713-14 (1972); *In re HTC Corp*., 889 F.3d 1349, 1357-58 (Fed. Cir. 2018), cert. *denied*, 139 S. Ct. 1271 (2019).

## IV. BACKGROUND

### A. TNF and TNF Receptors

19. Tumor necrosis factor ("TNF") is a cell-signaling protein involved in various biological effects that include the regulation of immune response, inflammation, and other processes. Scientists first identified it as a biological factor that was toxic to tumor cells; hence the name "tumor necrosis factor." The body's overproduction of TNF is also implicated in various autoimmune diseases and other inflammatory disorders.

20. TNF's biological effects can be mediated via specific TNF receptors on the membranes of certain cells. Such TNF receptors can specifically bind to TNF. This binding can trigger reactions inside the cell, which can give rise to a number of different responses, including inflammation, cell growth, and cell death.

21. The TNF receptors include: an extracellular region that binds to its ligand, TNF; a transmembrane region that anchors the receptor onto the cell membrane; and an intracellular region that provides signaling inside the cell. In the body, using natural biological processes, and in the lab, using biochemical techniques, the TNF-binding extracellular region can be cleaved from the cell membrane, leaving a TNF-binding soluble fragment of the TNF receptor.

22. Scientists knew, at the time of the filing of the Patents-In-Suit, that there were two cell-membrane-bound receptors specific to human TNF. One of these receptors was sometimes referred to as the human "p75 TNF receptor," and the other as the human "p55 TNF receptor." The p75 TNF receptor protein has an apparent molecular weight of about 75 kilodaltons on a non-reducing SDS-polyacrylamide gel; the p55 TNF receptor has an apparent molecular weight of about 55 kilodaltons.

**B. Immunex's Investment in Enbrel® (etanercept)**

23. Etanercept, the active ingredient in Enbrel®, is a dimeric fusion protein consisting of the extracellular ligand-binding portion of the human 75 kilodalton (p75) tumor necrosis factor receptor linked to the Fc portion of human IgG1. The Fc component of etanercept contains the CH2, the CH3, and hinge, but not the CH1 domain of IgG1. Etanercept is produced by recombinant DNA technology in a Chinese hamster ovary (CHO) mammalian cell expression system.

24. By binding to and inhibiting TNF from interacting with TNF receptors, etanercept can reduce certain inflammatory responses implicated in certain conditions such as rheumatoid arthritis, psoriasis, psoriatic arthritis, and others.

25. The FDA has approved Enbrel® for the following indications: rheumatoid arthritis, polyarticular juvenile idiopathic arthritis, psoriatic arthritis, ankylosing spondylitis, and plaque psoriasis. At the time of its first approval, and since, scientists and physicians have heralded Enbrel® as a major advance in treating these disorders.

26. Immunex conducted Phase I testing to determine whether Enbrel® was safe to administer to patients with rheumatoid arthritis; results published in 1993 indicated that it was. Immunex then conducted Phase II testing to begin determining whether Enbrel® improved symptoms of rheumatoid arthritis; results indicating that it did improve symptoms were published in 1996. Immunex conducted Phase III testing and invested a substantial amount of time and resources testing Enbrel® to demonstrate that it was safe and effective for certain disorders. Immunex invested considerable time and resources, and took considerable risk, in conducting these tests and obtaining their results.

27. Based on the results of clinical testing in rheumatoid arthritis, Immunex filed Biologic License Application ("BLA") No. 103795. As a result, in November 1998, the FDA first approved Enbrel®, pursuant to BLA No. 103795, for treating moderate to severe rheumatoid arthritis. Immunex holds the rights to BLA No. 103795.

28. Immunex's further clinical testing revealed that Enbrel® was safe and effective to treat certain additional conditions. Based on Immunex's further clinical testing, Immunex filed supplements to BLA No. 103795, requesting that the FDA approve Enbrel® for certain additional indications. As a result, the FDA approved Enbrel® for treating polyarticular juvenile idiopathic arthritis in 1999, psoriatic arthritis in 2002, ankylosing spondylitis in 2003, and plaque psoriasis in 2004. These approvals are the direct result of Immunex's very significant investments in the development and clinical trials of Enbrel®.

### C. Bioepis's Knowledge of the Patents-In-Suit, Its Etanercept Biosimilar, and Its Abbreviated BLA

29. As alleged herein, Roche's '182 Patent issued the year before Bioepis's formation, in 2011, and Roche's '522 Patent issued in April 2012. Immunex's '549 Patent issued in March 2005, Immunex's '124 Patent issued in August 2005, and Immunex's '557 Patent issued in January 2007. Each of the Immunex Patents issued several years before Bioepis's formation. In the context of the relevant circumstances here, Bioepis was either aware of each of these patents or was willfully blind to their existence.

30. According to its website, Bioepis is part of the Samsung Group. Bioepis's website states that its first six targets for biosimilar drugs were "worth up to 52.9 billion USD in the global market, with an average growth rate of 21% per year. The size is estimated to mark 22.9 billion USD by 2020." Given the size of that market, it is reasonable to infer that before and while undertaking to develop a biosimilar, Bioepis would determine whether and what

patents protected the innovative drug Bioepis sought to target. Consistent with that inference, Bioepis's website advises that Bioepis was aware that the manufacture, use, offer for sale, sale, or importation of its biosimilars might be prohibited by patents: "Biosimilars can be manufactured when the original product's patent expires." http://www.samsungbioepis.com/en/newsroom/detail/Samsung-Bio-Business-Possible-Recreation-of-the-Semiconductor-Legend.html.

31. Based on the circumstances, it is reasonable to infer that Bioepis was aware, or at least willfully blind to the existence, of each of the Patents-In-Suit during the development or FDA approval process for Bioepis's etanercept biosimilar product.

32. Bioepis is piggybacking on the fruits of Immunex/AML and Roche's trailblazing efforts. Bioepis admits it has developed an etanercept biosimilar product that has the identical primary amino acid sequence as in Immunex's Enbrel®. D.I. 70 at ¶ 32.

33. On information and belief, Bioepis submitted BLA 761066 referencing Immunex's Enbrel® and seeking FDA approval under 42 U.S.C. § 262(k) to engage in the commercial manufacture, use or sale of Bioepis's etanercept biosimilar product before the expiration of the Patents-In-Suit.

34. According to the FDA-approved label, Bioepis's etanercept biosimilar product, etanercept-ykro, like Immunex's Enbrel®, "is a dimeric fusion protein consisting of the extracellular ligand-binding portion of the human 75 kilodalton (p75) tumor necrosis factor receptor (TNFR) linked to the Fc portion of human IgG1. The Fc component of etanercept-ykro contains the CH2 domain, the CH3 domain and hinge region, but not the CH1 domain of IgG1. Etanercept-ykro is produced by recombinant DNA technology in a Chinese hamster ovary

(CHO) mammalian cell expression system." On information and belief, Bioepis's etanercept biosimilar specifically binds human TNF.

35. As noted in an April 25, 2019 review by the FDA, "the Agency determined that: SB4 [(i.e., Eticovo)] is highly similar to US-licensed Enbrel, notwithstanding minor differences in clinically inactive components." https://www.accessdata.fda.gov/drugsatfda_docs/nda/2019/761066Orig1s000SumR.pdf. As such, and on information and belief, Bioepis's manufacturing processes do not materially change the etanercept active ingredient of its etanercept biosimilar product.

36. On information and belief, in seeking FDA approval for its etanercept biosimilar product, Bioepis extensively and explicitly relied on the clinical trials data that Immunex had invested in and developed when applying for and securing FDA approval for Enbrel®.

37. On information and belief, Bioepis copied the FDA-approved label for Immunex's Enbrel® in seeking and receiving approval for its etanercept biosimilar product. Bioepis's etanercept biosimilar product, like Immunex's Enbrel®, has been approved for five indications: treating rheumatoid arthritis, polyarticular juvenile idiopathic arthritis, psoriatic arthritis, ankylosing spondylitis, and plaque psoriasis. In addition, the route of administration of Bioepis's etanercept biosimilar is the same as that of Immunex's Enbrel®, and the approved dosage form and strength of Bioepis's etanercept biosimilar represents a subset of the approved forms and strengths of Immunex's Enbrel®.

**D. Bioepis's Failure to Comply with the BPCIA**

38. The BPCIA provides that "[w]hen a subsection (k) applicant submits an application under subsection (k), such applicant shall provide to the persons described in clause (ii), subject to the terms of this paragraph, confidential access to the information required

to be produced pursuant to paragraph (2) and any other information that the subsection (k) applicant determines, in its sole discretion, to be appropriate (referred to in this subsection as the 'confidential information')." 42 U.S.C. § 262(*l*)(1)(B).

39. The referenced paragraph (2) provides that "[n]ot later than 20 days after the Secretary notifies the subsection (k) applicant that the application has been accepted for review, the subsection (k) applicant—

> (A) shall provide to the reference product sponsor a copy of the application submitted to the Secretary under subsection (k), and such other information that describes the process or processes used to manufacture the biological product that is the subject of such application; and
>
> (B) may provide to the reference product sponsor additional information requested by or on behalf of the reference product sponsor."

42 U.S.C. § 262(*l*)(2).

40. Bioepis has failed to provide to Immunex any of the information specified by 42 U.S.C. § 262(*l*)(2), including the application and information required under § 262(*l*)(2)(A). Such failure removed any limits on Plaintiffs' ability to bring an action for a declaration of infringement, validity, or enforceability of any patent that claims Bioepis's biosimilar etanercept or the use thereof. 42 U.S.C. § 262(*l*)(9)(C); 28 U.S.C. § 2201(b). Moreover, in light of Bioepis's failure to provide the specified information, Bioepis's submission of a BLA referencing Immunex's Enbrel® is an act of infringement of any patent that could be identified pursuant to 42 U.S.C. § 262(*l*)(3)(A). *See* 35 U.S.C. § 271(e)(2)(C)(ii).

41. The BPCIA requires that "[t]he subsection (k) applicant shall provide notice to the reference product sponsor not later than 180 days before the date of the first commercial marketing of the biological product licensed under subsection (k)." 42 U.S.C. § 262(*l*)(8)(A).

42. Bioepis has not yet provided Immunex the notice of commercial marketing that 42 U.S.C. § 262(*l*)(8)(A) requires. Based on Bioepis's failure to provide Immunex with the

application and information required under § 262(*l*)(2)(A), it is reasonable to infer that Bioepis might not provide notice to Immunex in accordance with § 262(*l*)(8)(A). Bioepis should be prohibited from beginning commercial marketing of its biosimilar product for at least 180 days from the date Bioepis provides such notice to Immunex.

## V. THE PATENTS-IN-SUIT

### A. The '182 and '522 Patents (Roche Patents)

43. In the late 1980s, Roche and Immunex scientists were early pioneers in isolating, characterizing, cloning, and sequencing p55 and p75 versions of the human TNF receptors, respectively.

44. Roche scientists were the first to publish the human p55 TNF receptor gene's amino acid sequence. *See* Loetscher *et al.*, "Molecular Cloning and Expression of the Human 55 kd Tumor Necrosis Factor Receptor," *Cell*, 61:351-359 (April 20, 1990).

45. In May 1990, Immunex scientists were the first to publish the p75 TNF receptor gene's amino acid sequence. *See* Smith *et al.*, "A Receptor for Tumor Necrosis Factor Defines an Unusual Family of Cellular and Viral Proteins," *Science* 248:1019-1023 (1989). Shortly thereafter, Roche scientists also published the p75 receptor's amino acid sequence, confirming the results published in Smith. Dembic *et al.*, "Two human TNF receptors have similar extracellular, but distinct intracellular, domain sequences," *Cytokine* 2(4):231-237 (1989).

46. On August 31, 1990, Roche scientists filed European Patent Application No. 90116707.2, which disclosed and taught the novel concept of fusing the extracellular fragment of the TNF receptors with a portion of the human immunoglobulin heavy chain (*i.e.*, all of the domains of the constant region of a human immunoglobulin IgG heavy chain other than the first domain of said constant region). These Roche scientists also filed a United States patent application on September 10, 1990, which claimed priority to said European patent application.

47. The Roche Patents both issued from applications that claim priority to the European patent application filed on August 31, 1990.

48. The ’182 Patent is directed to a fusion protein incorporating a TNF-binding portion of the p75 TNF receptor and covers etanercept. The ’522 Patent is directed to nucleic acids, host cells, and methods of using such nucleic acids and host cells to make the p75 TNF receptor fusion protein. Both Roche Patents could have been identified in Immunex’s list pursuant to 42 U.S.C. § 262(*l*)(3)(A) had Bioepis complied with § 262(*l*)(2)(A).

**B. <u>The ’549, ’124, and ’557 Patents (Immunex Patents)</u>**

49. In developing etanercept as a therapeutic, Immunex developed various improvements to the method of manufacturing etanercept and has obtained patents covering such methods.

50. The ’549 Patent, filed in March 2003, is directed to methods of increasing the production of a polypeptide (such as etanercept) by culturing a mammalian cell line that has been genetically engineered to produce the polypeptide in a production phase temperature of less than 37$^{o}$C and in a medium where a xanthine derivative or a hybrid polar compound is added during the production phase.

51. The ’124 Patent, filed in August 2002, is directed to methods of increasing the production of a recombinant protein (such as etanercept) by culturing a Chinese Hamster Ovary (“CHO”) cell engineered to produce the recombinant protein in a culture medium and adding a feed solution comprising a phosphate compound in an amount sufficient to attain a certain culture concentration of phosphate.

52. The ’557 Patent, filed in February 2002, is directed to methods of increasing the yield and recovery of a desired conformation of a recombinant soluble form of a p75 TNF-

receptor (including etanercept) that has higher binding affinity to its ligand than an undesired conformation by contacting a preparation of the recombinant soluble form of a p75 TNF-receptor with a reduction/oxidation coupling reagent.

**COUNT 1: FAILURE TO SUPPLY NOTICE OF COMMERCIAL MARKETING UNDER 42 U.S.C. § 262(*l*)(8)(A)**

53. Paragraphs 1-52 are incorporated by reference as if fully set forth herein.

54. The BPCIA provides that "[t]he subsection (k) applicant shall provide notice to the reference product sponsor not later than 180 days before the date of the first commercial marketing of the biological product licensed under subsection (k)." 42 U.S.C. § 262(*l*)(8)(A).

55. Bioepis has not provided notice to Immunex pursuant to 42 U.S.C. § 262(*l*)(8)(A); by its terms, that subsection operates to bar Bioepis from commercial marketing pending, at a minimum, such notice, followed by 180 days.

56. On information and belief, Bioepis is prepared to begin to use, offer, for sale, and sell in the United States, and import into the United States, its etanercept biosimilar product.

57. Immunex is entitled to injunctive relief preventing Bioepis from commercial marketing consistent with the notice period provided by that statute.

**COUNT 2: INFRINGEMENT OF THE '182 PATENT UNDER 35 U.S.C. § 271(e)(2)(C)(ii)**

58. Paragraphs 1-57 are incorporated by reference as if fully set forth herein.

59. The United States Patent and Trademark Office ("USPTO") duly and legally issued the '182 Patent, titled "Human TNF Receptor Fusion Protein," on November 22, 2011. A true and correct copy of the '182 Patent is attached to this Complaint as Exhibit 1.

60. Claims of the '182 Patent cover etanercept and pharmaceutical compositions that are made from etanercept. Thus, the '182 Patent could have been identified in Immunex's list pursuant to 42 U.S.C. § 262(*l*)(3)(A) had Bioepis complied with § 262(*l*)(2)(A).

61. On information and belief, Bioepis infringed claims of the '182 Patent by submitting a BLA referencing Immunex's Enbrel® and seeking FDA approval under 42 U.S.C. § 262(k) to engage in the commercial manufacture, use, or sale of Bioepis's etanercept biosimilar product before the expiration of the '182 Patent.

62. On information and belief, Bioepis has known of the '182 Patent since Bioepis was founded or has been willfully blind to its existence and contents since then. Despite such knowledge, Bioepis nonetheless filed its BLA with the FDA, seeking approval from the FDA to engage in the commercial manufacture, use or sale of Bioepis's etanercept biosimilar product before the expiration of the '182 Patent and in violation of Immunex/AML and Roche's patent rights.

63. Immunex/AML and Roche are entitled to a judgment that Bioepis has infringed one or more claims of the '182 Patent by submitting a BLA referencing Immunex's Enbrel® and seeking FDA approval under 42 U.S.C. § 262(k) to engage in the commercial manufacture, use, or sale of Bioepis's etanercept biosimilar product before the expiration of the '182 Patent.

64. Immunex/AML and/or Roche would be irreparably harmed if Bioepis is not enjoined from the commercial manufacture, use, offer for sale, or sale within the United States, or importation into the United States of Bioepis's FDA approved etanercept biosimilar product. Immunex/AML and Roche do not have an adequate remedy at law and are entitled to injunctive relief preventing Bioepis from such infringement of one or more claims of the '182 Patent.

**COUNT 3: DECLARATORY JUDGMENT OF INFRINGEMENT OF THE '182 PATENT UNDER 35 U.S.C. § 271(a)**

65. Paragraphs 1-64 are incorporated by reference as if fully set forth herein.

66. On information and belief, Bioepis has sought and obtained FDA approval of Bioepis's biosimilar etanercept product under 42 U.S.C. § 262(k) by reference to Immunex's

Enbrel®, and now holds the biological license granted by FDA for Bioepis's biosimilar etanercept product.

67. On information and belief, Bioepis intends to and will, immediately after 180 days from notice pursuant to 42 U.S.C. § 262(*l*)(8)(A), begin to use, offer for sale, or sell within the United States, or import into the United States, Bioepis's etanercept biosimilar product, which would constitute infringement of one or more claims of the '182 Patent under 35 U.S.C. § 271(a).

68. An actual controversy has arisen and now exists between the parties concerning whether Bioepis's using, offering to sell, or selling within the United States, or importing into the United States, its etanercept biosimilar product has infringed and/or will infringe one or more claims of the '182 Patent.

69. Immunex/AML and Roche are entitled to a declaratory judgment that Bioepis has infringed and/or would infringe one or more claims of the '182 Patent by making, using, offering to sell, or selling within the United States, or importing into the United States, Bioepis's etanercept biosimilar product before the expiration of the '182 Patent.

70. Immunex/AML and/or Roche would be irreparably harmed if Bioepis is not enjoined from infringing one or more claims of the '182 Patent. Immunex/AML and Roche do not have an adequate remedy at law and are entitled to injunctive relief prohibiting Bioepis from making, using, offering to sell, or selling within the United States, or importing into the United States, Bioepis's etanercept biosimilar product before the expiration of the '182 Patent.

**COUNT 4: INFRINGEMENT OF THE '522 PATENT UNDER 35 U.S.C. § 271(e)(2)(C)(ii)**

71. Paragraphs 1-57 are incorporated by reference as if fully set forth herein.

72. The USPTO duly and legally issued the '522 Patent, titled "Human TNF Receptor," on April 24, 2012. A true and correct copy of the '522 Patent is attached to this Complaint as Exhibit 2.

73. Claims of the '522 Patent cover, among other things, methods of making etanercept and certain materials used in such methods. Thus, the '522 Patent could have been identified in Immunex's list pursuant to 42 U.S.C. § 262(*l*)(3)(A) had Bioepis complied with § 262(*l*)(2)(A).

74. On information and belief, Bioepis infringed claims of the '522 Patent by submitting a BLA referencing Immunex's Enbrel®, seeking FDA approval under 42 U.S.C. § 262(k) to engage in the commercial manufacture, use, or sale of Bioepis's etanercept biosimilar product before the expiration of the '522 Patent.

75. On information and belief, Bioepis has known of the '522 Patent since its issuance or has been willfully blind to its existence and contents since then. Despite such knowledge, Bioepis nonetheless filed its BLA with the FDA, seeking approval from the FDA to engage in the commercial manufacture, use or sale of Bioepis's etanercept biosimilar product, before the expiration of the '522 Patent and in violation of Immunex/AML and Roche's patent rights.

76. Immunex/AML and Roche are entitled to a judgment that Bioepis has infringed one or more claims of the '522 Patent by submitting a BLA referencing Immunex's Enbrel® and seeking FDA approval under 42 U.S.C. § 262(k) to engage in the commercial manufacture, use, or sale of Bioepis's etanercept biosimilar product before the expiration of the '522 Patent.

77. Immunex/AML and/or Roche would be irreparably harmed if Bioepis is not enjoined from the commercial manufacture, use, offer for sale, or sale within the United States,

or importation into the United States of Bioepis's FDA approved etanercept biosimilar product. Immunex/AML and Roche do not have an adequate remedy at law and are entitled to injunctive relief preventing Bioepis from such infringement of one or more claims of the '522 Patent.

**COUNT 5: DECLARATORY JUDGMENT OF INFRINGEMENT OF THE '522 PATENT UNDER 35 U.S.C. § 271(g)**

78. Paragraphs 1-57, 71-77 are incorporated by reference as if fully set forth herein.

79. On information and belief, Bioepis intends to and will, immediately after 180 days from notice pursuant to 42 U.S.C. § 262(*l*)(8)(A), begin to import into the United States, and offer to sell, sell, and use within the United States, Bioepis's etanercept biosimilar product, which would constitute infringement of one or more claims of the '522 Patent under 35 U.S.C. § 271(g) because Bioepis's etanercept biosimilar product is made by the claimed process.

80. The etanercept made by Bioepis's process that infringes the '522 Patent is the essential active ingredient of Bioepis's etanercept biosimilar product. On information and belief, there is no subsequent process that materially changes that active ingredient, including during any fill and finish of the biosimilar product.

81. An actual controversy has arisen and now exists between the parties concerning whether Bioepis's importing into the United States, or offering to sell, selling, or using within the United States (irrespective of where manufacturing occurred), its etanercept biosimilar product, before the expiration of the '522 Patent, has infringed and/or will infringe one or more claims of the '522 Patent.

82. Immunex/AML and Roche are entitled to a declaratory judgment that Bioepis has infringed and/or will infringe one or more claims of the '522 Patent by making, using, offering to sell, or selling within the United States, or importing into the United States, Bioepis's etanercept biosimilar product before the expiration of the '522 Patent.

83. Immunex/AML and/or Roche will be irreparably harmed if Bioepis is not enjoined from infringing one or more claims of the '522 Patent. Immunex/AML and Roche do not have an adequate remedy at law and are entitled to injunctive relief preventing Bioepis from making, using, offering to sell, or selling within the United States, or importing into the United States, Bioepis's etanercept biosimilar product before the expiration of the '522 Patent.

84. The following paragraphs (85-105) are alleged only by Immunex/AML.

**COUNT 6: INFRINGEMENT OF THE '549 PATENT UNDER 35 U.S.C. § 271(e)(2)(C)(ii)**

85. Paragraphs 1-57 are incorporated by reference as if fully set forth herein.

86. The USPTO duly and legally issued the '549 Patent, titled "Methods for Increasing Polypeptide Production," on March 29, 2005. A true and correct copy of the '549 Patent is attached to this Complaint as Exhibit 3.

87. Claims of the '549 Patent cover methods of increasing the production of a polypeptide (such as etanercept) by culturing a mammalian cell line that has been genetically engineered to produce the polypeptide in a production phase temperature of less than 37°C and in a medium where a xanthine derivative or a hybrid polar compound is added during the production phase. Thus, the '549 Patent could have been identified in Immunex's list pursuant to 42 U.S.C. § 262*(l)*(3)(A) had Bioepis complied with § 262(*l*)(2)(A).

88. On information and belief, Bioepis has infringed the '549 Patent by submitting a BLA referencing Immunex's Enbrel®, seeking FDA approval under 42 U.S.C. § 262(k) to engage in the commercial manufacture, use, or sale of Bioepis's etanercept biosimilar product before the expiration of the '549 Patent.

89. On information and belief, Bioepis has known of the '549 Patent, which issued on March 29, 2005, since Bioepis was founded or was at least willfully blind to its existence and

contents. Despite such knowledge or willful blindness, Bioepis nonetheless filed its BLA with the FDA, seeking approval under 42 U.S.C. § 262(k) to engage in the commercial manufacture, use, or sale of Bioepis's etanercept biosimilar product before the expiration of the '549 Patent and in violation of Immunex/AML's patent rights.

90. Immunex/AML are entitled to a judgment that Bioepis has infringed one or more claims of the '549 Patent by submitting a BLA referencing Immunex's Enbrel® and seeking FDA approval under 42 U.S.C. § 262(k) to engage in the commercial manufacture, use, or sale of Bioepis's etanercept biosimilar product before the expiration of the '549 Patent.

91. Immunex/AML would be irreparably harmed if Bioepis is not enjoined from the commercial manufacture, use, offer for sale, or sale within the United States, or importation into the United States of Bioepis's FDA approved etanercept biosimilar product. Immunex/AML do not have an adequate remedy at law and are entitled to injunctive relief preventing Bioepis from such infringement of one or more claims of the '549 Patent.

**COUNT 7: INFRINGEMENT OF THE '124 PATENT UNDER 35 U.S.C. § 271(e)(2)(C)(ii)**

92. Paragraphs 1-57 are incorporated by reference as if fully set forth herein.

93. The USPTO duly and legally issued the '124 Patent, titled "Feeding Strategies for Cell Culture," on August 2, 2005. A true and correct copy of the '124 Patent is attached to this Complaint as Exhibit 4.

94. Claims of the '124 Patent cover methods of increasing the production of a recombinant protein (such as etanercept) by culturing a Chinese Hamster Ovary ("CHO") cell engineered to produce the recombinant protein in a culture medium and adding a feed solution comprising a phosphate compound of a certain concentration. Thus, the '124 Patent could have

been identified in Immunex's list pursuant to 42 U.S.C. § 262*(l)*(3)(A) had Bioepis complied with § 262(*l*)(2)(A).

95. On information and belief, Bioepis has infringed the '124 Patent by submitting a BLA referencing Immunex's Enbrel®, seeking FDA approval under 42 U.S.C. § 262(k) to engage in the commercial manufacture, use, or sale of Bioepis's etanercept biosimilar product before the expiration of the '124 Patent.

96. On information and belief, Bioepis has known of the '124 Patent, which issued on August 2, 2005, since Bioepis was founded or was at least willfully blind to its existence and contents. Despite such knowledge or willful blindness, Bioepis nonetheless filed its BLA with the FDA, seeking approval under 42 U.S.C. § 262(k) to engage in the commercial manufacture, use, or sale of Bioepis's etanercept biosimilar product before the expiration of the '124 Patent and in violation of Immunex/AML's patent rights.

97. Immunex/AML are entitled to a judgment that Bioepis has infringed one or more claims of the '124 Patent by submitting a BLA referencing Immunex's Enbrel® and seeking FDA approval under 42 U.S.C. § 262(k) to engage in the commercial manufacture, use, or sale of Bioepis's etanercept biosimilar product before the expiration of the '124 Patent.

98. Immunex/AML would be irreparably harmed if Bioepis is not enjoined from the commercial manufacture, use, offer for sale, or sale within the United States, or importation into the United States of Bioepis's FDA approved etanercept biosimilar product. Immunex/AML do not have an adequate remedy at law and are entitled to injunctive relief preventing Bioepis from such infringement of one or more claims of the '124 Patent.

**COUNT 8: INFRINGEMENT OF THE '557 PATENT UNDER 35 U.S.C. § 271(e)(2)(C)(ii)**

99. Paragraphs 1-57 are incorporated by reference as if fully set forth herein.

100. The USPTO duly and legally issued the '557 Patent, titled "Increased Recovery of Active Proteins," on January 2, 2007. A true and correct copy of the '557 Patent is attached to this Complaint as Exhibit 5.

101. Claims of the '557 Patent cover methods of increasing the yield and recovery of a desired conformation of a recombinant soluble form of a p75 TNF-receptor (including etanercept) that has higher binding affinity to its ligand than an undesired conformation by contacting a preparation of the recombinant soluble form of a p75 TNF-receptor with a reduction/oxidation coupling reagent. Thus, the '557 Patent could have been identified in Immunex's list pursuant to 42 U.S.C. § 262*(l)*(3)(A) had Bioepis complied with § 262(*l*)(2)(A).

102. On information and belief, Bioepis has infringed the '557 Patent by submitting a BLA referencing Immunex's Enbrel®, seeking FDA approval under 42 U.S.C. § 262(k) to engage in the commercial manufacture, use, or sale of Bioepis's etanercept biosimilar product before the expiration of the '557 Patent.

103. On information and belief, Bioepis has known of the '557 Patent, which issued on January 2, 2007, since Bioepis was founded or was at least willfully blind to its existence and contents. Despite such knowledge or willful blindness, Bioepis nonetheless filed its BLA with the FDA, seeking approval under 42 U.S.C. § 262(k) to engage in the commercial manufacture, use, or sale of Bioepis's etanercept biosimilar product before the expiration of the '557 Patent and in violation of Immunex/AML's patent rights.

104. Immunex/AML are entitled to a judgment that Bioepis has infringed one or more claims of the '557 Patent by submitting a BLA referencing Immunex's Enbrel® and seeking FDA approval under 42 U.S.C. § 262(k) to engage in the commercial manufacture, use, or sale of Bioepis's etanercept biosimilar product before the expiration of the '557 Patent.

105. Immunex/AML would be irreparably harmed if Bioepis is not enjoined from the commercial manufacture, use, offer for sale, or sale within the United States, or importation into the United States of Bioepis's FDA approved etanercept biosimilar product. Immunex/AML do not have an adequate remedy at law and are entitled to injunctive relief preventing Bioepis from such infringement of one or more claims of the '557 Patent.

**PRAYER FOR RELIEF**

WHEREFORE, Roche (with respect to the '182 and '522 Patents) and Immunex/AML (with respect to all Patents-In-Suit) respectfully request that this Court enter judgment in their favor against Bioepis and grant the following relief:

**Temporary and preliminary injunctive relief**

A. A temporary restraining order enjoining the commercial making, using, offering for sale, and selling in the United States, or importing into the United States, of Bioepis's etanercept biosimilar product pending briefing and this Court's decision on a motion for preliminary injunction;

B. A preliminary injunction enjoining the commercial making, using, offering for sale, and selling in the United States, or importing into the United States, of Bioepis's etanercept biosimilar product until no less than 180 days after Bioepis provides the notice of commercial marketing that the BPCIA requires; and

C. A preliminary injunction enjoining the commercial making, using, offering for sale, and selling in the United States, or importing into the United States, of Bioepis's etanercept biosimilar product pending a final determination in this matter as to infringement, validity, and enforceability of the asserted claims of the Patents-In-Suit.

**Judgments and permanent injunctive relief for infringement under 35 U.S.C. § 271(e)(2)(C)(ii)**

A. A judgment under 35 U.S.C. § 271(e)(2)(C)(ii) that by submitting to the FDA Bioepis's BLA to obtain approval of Bioepis's etanercept biosimilar product, Bioepis has infringed one or more claims of each of the Patents-In-Suit;

B. Based on that judgment, a permanent injunction against the commercial manufacture, use, offer to sell, or sale within the United States or importation into the United States of Bioepis's etanercept biosimilar product before the expiration of the last to expire of the Patents-In-Suit; and

C. A declaration that this is an exceptional case and awarding attorneys' fees and costs pursuant to 35 U.S.C. § 285.

**Judgments, and relief, for infringement under 35 U.S.C. § 271(a)**

A. A judgment that Bioepis has infringed or will infringe one or more claims of the '182 Patent by making, using, offering for sale, or selling within the United States, or importing into the United States, Bioepis's etanercept biosimilar product during the term of the '182 Patent;

B. Based on that judgment, a permanent injunction against future infringement by Bioepis, as well as by its officers, employees, agents, representatives, affiliates, assignees, successors, and affiliates, and all persons acting on behalf of, at the direction of, or in active concert with it, until the '182 Patent expires; and

C. A declaration that this is an exceptional case and awarding attorneys' fees and costs pursuant to 35 U.S.C. § 285.

**Judgments, and relief, for infringement under 35 U.S.C. § 271(g)**

A. A judgment that Bioepis has infringed or will infringe one or more claims of the '522 Patent by importing into the United States, or offering to sell, selling, or using its etanercept

biosimilar product within the United States during the term of the '522 Patent;

B. Based on that judgment, a permanent injunction against future infringement by Bioepis, as well as by its officers, employees, agents, representatives, affiliates, assignees, successors, and affiliates, and all persons acting on behalf of, at the direction of, or in active concert with it, until the '522 Patent expires; and

C. A declaration that this is an exceptional case and awarding attorneys' fees and costs pursuant to 35 U.S.C. § 285.

**On all counts: Other relief**

A. On all counts, such other relief as this Court may deem just, necessary, or proper pursuant to 28 U.S.C. § 2202.

**DEMAND FOR A JURY TRIAL**

Immunex/AML and Roche hereby demand a jury trial on all issues so triable.

Dated: December 23, 2019

*s/ Liza M. Walsh*

Liza M. Walsh
Marc D. Haefner
Christine I. Gannon
WALSH PIZZI O'REILLY FALANGA LLP
Three Gateway Center
100 Mulberry Street, 15th Floor
Newark, New Jersey 07102
(973) 757-1100

OF COUNSEL:

David T. Pritikin
Steven J. Horowitz
Richard M. Chen
SIDLEY AUSTIN LLP
One South Dearborn

*s/ Charles H. Chevalier*

David E. De Lorenzi
Charles H. Chevalier
Gibbons P.C.
One Gateway Center
Newark, New Jersey 07102-5310
(973) 596-4500

OF COUNSEL:

David I. Berl
Aaron Maurer
Thomas S. Fletcher
WILLIAMS & CONNOLLY LLP
725 Twelfth St. NW
Washington, D.C. 20005
(202) 434-5000

Chicago, Illinois 60603
(312) 853-7000

Vernon M. Winters
Sue Wang
SIDLEY AUSTIN LLP
555 California Street, Suite 2000
San Francisco, California 94104
(415) 772-1200

Jeffrey P. Kushan
Peter S. Choi
SIDLEY AUSTIN LLP
1501 K Street N.W.
Washington, D.C. 20005
(202) 736-8700

Samuel N. Tiu
Brooke S. Böll
SIDLEY AUSTIN LLP
555 West Fifth Street
Los Angeles, California 90013
(213) 896-6000

Wendy Whiteford
J. Drew Diamond
Dennis Smith
Joseph E. Lasher
James A. High, Jr.
AMGEN INC.
One Amgen Center Drive
Thousand Oaks, CA 91320-1789
(805) 447-1000

*Attorneys for Immunex Corporation and Amgen Manufacturing, Limited*

*Attorneys for Hoffmann-La Roche Inc.*

**RULE 201.1 CERTIFICATION**

I hereby certify that the above-captioned matter is not subject to compulsory arbitration in that the Plaintiffs seek, *inter alia*, injunctive relief.

Dated: December 23, 2019

*s/ Liza M. Walsh*

Liza M. Walsh
Marc D. Haefner
Christine I. Gannon
WALSH PIZZI O'REILLY FALANGA LLP
Three Gateway Center
100 Mulberry Street, 15th Floor
Newark, New Jersey 07102
(973) 757-1100

OF COUNSEL:

David T. Pritikin
Steven J. Horowitz
Richard M. Chen
SIDLEY AUSTIN LLP
One South Dearborn
Chicago, Illinois 60603
(312) 853-7000

Vernon M. Winters
Sue Wang
SIDLEY AUSTIN LLP
555 California Street, Suite 2000
San Francisco, California 94104
(415) 772-1200

Jeffrey P. Kushan
Peter S. Choi
SIDLEY AUSTIN LLP
1501 K Street N.W.
Washington, D.C. 20005
(202) 736-8700

Samuel N. Tiu
Brooke S. Böll
SIDLEY AUSTIN LLP
555 West Fifth Street

*s/ Charles H. Chevalier*

David E. De Lorenzi
Charles H. Chevalier
Gibbons P.C.
One Gateway Center
Newark, New Jersey 07102-5310
(973) 596-4500

OF COUNSEL:

David I. Berl
Aaron Maurer
Thomas S. Fletcher
WILLIAMS & CONNOLLY LLP
725 Twelfth St. NW
Washington, D.C. 20005
(202) 434-5000
*Attorneys for Hoffmann-La Roche Inc.*

Los Angeles, California 90013
(213) 896-6000

Wendy Whiteford
J. Drew Diamond
Dennis Smith
Joseph E. Lasher
James A. High, Jr.
AMGEN INC.
One Amgen Center Drive
Thousand Oaks, CA 91320-1789
(805) 447-1000

*Attorneys for Immunex Corporation and Amgen Manufacturing, Limited*

# EXHIBIT 1

US008063182B1

(12) **United States Patent**
**Brockhaus et al.**

(10) **Patent No.: US 8,063,182 B1**
(45) **Date of Patent: Nov. 22, 2011**

(54) **HUMAN TNF RECEPTOR FUSION PROTEIN**

(75) Inventors: **Manfred Brockhaus**, Bettingen (CH); **Reiner Gentz**, Rheinfelden (DE); **Dembic Zlatko**, Basel (CH); **Werner Lesslauer**, Basel (CH); **Hansruedi Lotscher**, Mohlin (CH); **Ernst-Jurgen Schlaeger**, Efringen-Kirchen (DE)

(73) Assignee: **Hoffman-LaRoche Inc.**, Nutley, NJ (US)

( * ) Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 0 days.

(21) Appl. No.: **08/444,790**

(22) Filed: **May 19, 1995**

**Related U.S. Application Data**

(60) Division of application No. 08/095,640, filed on Jul. 21, 1993, now Pat. No. 5,610,279, which is a continuation of application No. 07/580,013, filed on Sep. 10, 1990, now abandoned.

(30) **Foreign Application Priority Data**

| | | |
|---|---|---|
| Sep. 12, 1989 | (CH) | 3319/89 |
| Mar. 8, 1990 | (CH) | 746/90 |
| Apr. 20, 1990 | (CH) | 1347/90 |
| Aug. 31, 1990 | (EP) | 90116707 |

(51) **Int. Cl.**

| | |
|---|---|
| ***C07K 14/715*** | (2006.01) |
| ***A61P 29/00*** | (2006.01) |
| ***A61K 38/17*** | (2006.01) |
| ***C07K 19/00*** | (2006.01) |
| *C07H 21/04* | (2006.01) |

(52) **U.S. Cl.** ..... **530/350**; 514/12.2; 530/387.3; 536/23.5; 930/144

(58) **Field of Classification Search** ..... 530/324, 530/350; 424/192.1; 536/23.5
See application file for complete search history.

(56) **References Cited**

U.S. PATENT DOCUMENTS

| | | | | |
|---|---|---|---|---|
| 4,593,002 | A | | 6/1986 | Dulbecco |
| 4,675,285 | A | | 6/1987 | Clark et al. |
| 4,729,326 | A | | 3/1988 | Richter |
| 4,769,326 | A | | 9/1988 | Rutler |
| 4,770,995 | A | | 9/1988 | Rubin et al. |
| 4,816,567 | A | | 3/1989 | Cabilly et al. |
| 4,894,439 | A | | 1/1990 | Dorin et al. |
| 4,912,044 | A | | 3/1990 | Jacob et al. |
| 4,935,233 | A | | 6/1990 | Bell et al. |
| 4,948,875 | A | | 8/1990 | Tanaka et al. |
| 4,963,354 | A | | 10/1990 | Shepard et al. |
| 4,965,271 | A | | 10/1990 | Mandell et al. |
| 5,055,447 | A | | 10/1991 | Palladino et al. |
| 5,073,627 | A | | 12/1991 | Curtis et al. |
| 5,075,222 | A | | 12/1991 | Hannum et al. |
| 5,098,702 | A | | 3/1992 | Zimmerman et al. |
| 5,098,833 | A | | 3/1992 | Lasky et al. |
| 5,116,964 | A | * | 5/1992 | Capon et al. ..... 536/23.5 |
| 5,118,500 | A | | 6/1992 | Hanel et al. |
| 5,136,021 | A | | 8/1992 | Dembinski et al. |
| 5,155,027 | A | | 10/1992 | Sledziewski et al. |
| 5,211,945 | A | | 5/1993 | Wallach et al. |
| 5,223,395 | A | | 6/1993 | Gero |
| 5,225,538 | A | | 7/1993 | Capon et al. |
| 5,258,498 | A | | 11/1993 | Huston et al. |
| 5,264,416 | A | | 11/1993 | Park et al. |
| 5,270,038 | A | | 12/1993 | Klimpel et al. |
| 5,336,603 | A | | 8/1994 | Capon et al. |
| 5,344,915 | A | * | 9/1994 | LeMaire et al. ..... 530/350 |
| 5,350,683 | A | | 9/1994 | Sims et al. |
| 5,359,032 | A | | 10/1994 | Dayer et al. |
| 5,359,037 | A | | 10/1994 | Wallach et al. ..... 530/388.22 |
| 5,395,760 | A | * | 3/1995 | Smith et al. ..... 435/365 |
| 5,428,130 | A | | 6/1995 | Capon et al. |
| 5,447,851 | A | | 9/1995 | Beutler et al. |
| 5,455,165 | A | | 10/1995 | Capon et al. |
| 5,478,925 | A | | 12/1995 | Wallach et al. |
| 5,512,544 | A | | 4/1996 | Wallach et al. |
| 5,514,582 | A | | 5/1996 | Capon et al. |
| 5,599,905 | A | | 2/1997 | Mosley et al. |
| 5,605,690 | A | | 2/1997 | Jacobs et al. |
| 5,610,279 | A | | 3/1997 | Brockhaus et al. |
| 5,633,145 | A | | 5/1997 | Feldmann et al. |

(Continued)

FOREIGN PATENT DOCUMENTS

| | | |
|---|---|---|
| AU | 58976 | 1/1991 |

(Continued)

OTHER PUBLICATIONS

Miles JS, et al. Principles of DNA cloning. British Medical Journal. 1989 299:1019-1022.*
Olsson, I, et al., Isolation and charaterization of a tumor necrosis factor binding protein from urine. Eur J Haematol. 1989;42:270-275.*
Dembic et al, 1990. Cytokine. 2(4): 231-7.*
Chan et al. 2000, Science, 288: 2351-2354.*
Smith et al, 1989. Journal of Biological Chemistry. 14646-15652.*
Heller et al., JBC 265(10): 5708-5717 (1990).

(Continued)

*Primary Examiner* — Gary Nickol
*Assistant Examiner* — Zachary Howard
(74) *Attorney, Agent, or Firm* — Marshall, Gerstein & Borun LLP

(57) **ABSTRACT**

The present invention is concerned with non-soluble proteins and soluble or insoluble fragments thereof, which bind TNF, in homogeneous form, as well as their physiologically compatible salts, especially those proteins having a molecular weight of about 55 or 75 kD (non-reducing SDS-PAGE conditions), a process for the isolation of such proteins, antibodies against such proteins, DNA sequences which code for non-soluble proteins and soluble or non-soluble fragments thereof, which bind TNF, as well as those which code for proteins comprising partly of a soluble fragment, which binds TNF, and partly of all domains except the first of the constant region of the heavy chain of human immunoglobulins and the recombinant proteins coded thereby as well as a process for their manufacture using transformed pro- and eukaryotic host cells.

**36 Claims, 6 Drawing Sheets**

U.S. PATENT DOCUMENTS

| | | | | |
|---|---|---|---|---|
| 5,639,597 | A | | 6/1997 | Lauffer et al. |
| 5,695,953 | A | | 12/1997 | Wallach et al. |
| 5,705,364 | A | | 1/1998 | Etcheverry et al. |
| 5,712,155 | A | | 1/1998 | Smith et al. ..... 735/320.1 |
| 5,721,121 | A | | 2/1998 | Etcheverry et al. |
| 5,808,029 | A | | 9/1998 | Brockhaus et al. |
| 5,811,261 | A | * | 9/1998 | Wallach et al. ..... 435/69.1 |
| 5,863,786 | A | | 1/1999 | Feldmann et al. |
| 5,447,851 | A | | 7/1999 | Beutler et al. |
| 5,945,397 | A | | 8/1999 | Smith et al. |
| 5,981,701 | A | * | 11/1999 | Wallach et al. ..... 530/350 |
| RE36,755 | E | | 6/2000 | Smith et al. |
| 6,143,866 | A | | 11/2000 | Brewer et al. |
| 6,165,476 | A | | 12/2000 | Strom et al. |
| 6,201,105 | B1 | | 3/2001 | Smith et al. |
| 6,221,675 | B1 | | 4/2001 | Hauptmann et al. |
| 6,271,346 | B1 | | 8/2001 | Hauptmann et al. |
| 6,294,352 | B1 | | 9/2001 | Hauptmann et al. |
| 6,417,158 | B1 | | 7/2002 | Hauptmann et al. |
| 6,541,610 | B1 | | 4/2003 | Smith |
| 6,541,620 | B1 | | 4/2003 | Brewer et al. |
| 6,572,852 | B2 | | 6/2003 | Smith et al. |
| 6,858,409 | B1 | | 2/2005 | Thompson et al. |
| 7,253,264 | B1 | | 8/2007 | Lauffer et al. |
| 2003/0064480 | A1 | | 4/2003 | Lauffler et al. |

FOREIGN PATENT DOCUMENTS

| | | | |
|---|---|---|---|
| EP | 120694 | | 10/1984 |
| EP | 227110 | | 7/1987 |
| EP | 230574 | | 8/1987 |
| EP | 269455 | | 6/1988 |
| EP | 315 062 | | 10/1988 |
| EP | 308 378 | | 3/1989 |
| EP | 0 314 317 | | 5/1989 |
| EP | 325 224 | | 7/1989 |
| EP | 325262 | | 7/1989 |
| EP | 0334165 | | 9/1989 |
| EP | 393 438 | | 4/1990 |
| EP | 0 394 827 | | 10/1990 |
| EP | 398 327 | | 11/1990 |
| EP | 412 486 | | 2/1991 |
| EP | 414178 | | 2/1991 |
| EP | 418 014 | | 3/1991 |
| EP | 417563 | | 3/1991 |
| EP | 422 339 | | 4/1991 |
| EP | 433 900 | | 6/1991 |
| EP | 460846 | | 12/1991 |
| EP | 464 533 | | 1/1992 |
| EP | 471701 | | 2/1992 |
| EP | 526452 | | 2/1993 |
| EP | 526905 | | 2/1993 |
| EP | 0 567 566 | B1 | 11/1993 |
| EP | 568925 | | 11/1993 |
| EP | 606869 | | 7/1994 |
| GB | 2218101 | | 11/1989 |
| GB | 2 246 569 | | 2/1992 |
| JP | 61-293924 | | 12/1986 |
| JP | 61-293924 | A2 | 12/1986 |
| JP | 02-154695 | | 6/1990 |
| WO | WO 89/02922 | * | 4/1989 |
| WO | WO 89 09622 | | 10/1989 |
| WO | 9013575 | | 11/1990 |
| WO | WO 91/02078 | | 2/1991 |
| WO | 91/03553 | | 3/1991 |
| WO | WO 91/17184 | | 11/1991 |
| WO | WO 91/08298 | | 12/1991 |
| WO | WO 92/08495 | | 5/1992 |
| WO | WO 92/13095 | | 8/1992 |
| WO | WO 93/07863 | | 4/1993 |
| WO | WO 93/19777 | | 10/1993 |
| WO | WO 94/06476 | | 3/1994 |

OTHER PUBLICATIONS

Abstract by Heller et al., Napa Valley Conference (1989).
Olsson et al., Eur. J. Haematol., 42: 270-275 (1989).
Nophar et al., EMBO J., 9:3269-3278 (1990).
Evans, T.J. et al., J. Exp. Med. 180, pp. 2173-2179 (1994).
Traunecker, A. et al., Nature, 339, pp. 68-70 (1989).
Dembie, Z., et al., Cytokine, 2, pp. 231-237 (1990).
Byrn, et al., Nature, 344, pp. 667-670 (1990).
Peppel, K., et al., J. Exp. Med., 174, pp. 1483-1489 (1991).
Zettlmeissl, G., et al., DNA & Cell Biology, 9, pp. 347-353 (1990).
Loetscher et al, *J. of Bio. Chem*., 266(27), pp. 18324-18329, (1991).
Nature Biotechnology, 15 (1997) p. 13.
Fisher, et al., N. Engl. J. of Med. 334, pp. 1697-1702 (1996).
Hsu, et al., J. Biol. Chem. 268, pp. 16430-16436 (1993).
Mohler et al., Soluble Tumor NecrosisFfactor (TNF) Receptors are Effective Therapeutic Agents in Lethal Endotoxemia and Function Simultaneously as Both TNF Carriers and TNF Antagonists. J. Immunol., 151:1548-1561 (1993).
Sell, Immunology, Immunopathology and Immunity, 4th Edition, Elsevier Science Publishing Co., New York, 1987, at pp. 85-91.
Bringman, Monoclonal antibodies to human tumor necrosis factors alpha and beta: application for affinity purification, immunoassays, and as structural probes. Hybridoma, 6(5):489-507 (1987).
Paul et al., Fundamental Immunology, 2nd Edition, Paul, ed., Raven Press, New York, 1989, at pp. 679-701 and pp. 735-764.
Smith et al., A Receptor for Tumor Necrosis Factor Defines an Unusual Family of Cellular and Viral Proteins, *Science*, 248:1019-1023, (1990).
Wingfield et al., Tumour Necrosis Factor is a Compact Trimer, FEBS Lett. 211: 179-84 (1987).
Smith and Baglioni, The ActiveFform of Tumor Necrosis Factor is a Trimer J. Biological Chemistry, 262:6951-4 (1987).
Larsson and Mosbach, Affintiy Precipitation of Enzymes FEBS Lett. 98(2):333-338 (1979).
Irwin and Tipton, Chapter 22, "Affinity Precipitation Methods" in Methods in Mol. Bio., 59:217 (1996).
Kohno et al., Presentation 1495, poster 271 presented at American College of Rheumatology Annual Meeting, Nov. 13-17, 2005, San Diego, CA.
Klein, Immunology, 1st Edition, Klein ed., Blackwell Scientific Publications, Cambridge, MA 1990 at pp. 446-447.
Byrn et al., Biological properties of a CD4 Immunoadhesin, Nature, 344:667-670 (Apr. 1990).
Traunecker et al., Highly Efficient Neutralization of HIV with Recombinant CD-4-Immunoglobulin Molecules, Nature 339:68-70, 1989.
Capon, Designing CD4 Immunoadhesions for AIDS Therapy, Nature, 337:525-531 (1989).
Barone et al., Comparative Analysis of the Ability of Entanercept and Inflixamab to Lyse TNF-Expressing Cells in a Complement-Dependent fashion, Arthritis Rheum., v42(9) supplement, Sep. 1999.
Khare et al, Poster 715 presented at the Annual Meeting of the Society for Investigative Dermatology (SID), May 3-5, 2006, Philadelphia, PA.
Nesbitt, et al., "Mechanism of Action of Certolizumab Pegol (Cdp870): In Vitro Comparison With Other Anti-Tumor Necrosis Factor α Agents", *Inflamm Bowel Dis*, 13: 1323-1332 (Nov. 2007).
Aruffo et al., "CD44 Is the Principal Cell Surface Receptor for Hyaluronate," Cell 61:1303-1313 (1990).
Brennan et al., "Inhibitory Effects of TNF α Antibodies on Synovial Cell Interleukin-1 Production in Rheumatoid Arthritis," The Lancet 2(8657):244-247 (1989).
Monnat, "Molecular Analysis of Spontaneous Hypoxanthine Phosphoribosyltransferase Mutations in Thioguanine-resistant HL-60 Human Leukemia Cells," Cancer Res. 49:81-87 (1989).
Shalaby et al., "The Involvement of Human Tumor Necrosis Factors-α and -βin the Mixed Lymphocyte Reaction," J. Immunol. 141:449-503 (1988).
Watson et al., "A Homing Receptor—IgG Chimera as a Probe for Adhesive Ligands of Lymph Node High Endothelial Venules," J. Cell Biol. 110:2221-2229 (1990).
Gen Seq DBase Printout.
Stauber et al., "Human tumor necrosis factor-α receptor—purification by immunoaffinity chromatography and initial characterization", J. Bio. Chem. 263:19098-19104 (1988).
Seckinger et al., "Purification and biologic characterization of a sp cific tum r necrosis fact r α inhibitor", J. Bio. Chem. 264:11966-11973 (1989).

Engelmann et al., "A tumor necrosis factor-binding protein purified to homogeneity from human urine protects cells from tumor necrosis factor toxicity", J. Bio. Chem. 264:11974-11980 (1989).
Hohmann et al., "Two different cell types have different major r cept rs f r human tumor necr sis factor (TNFα)", J. Bio. Chem. 264:14927-14934 (1989).
Smith t al., "A receptor for tum r necrosis factor defines an unusual family of cellular and viral proteins", Scienc 248:1019-1023 (1990).
Heller et al., "Complementary DNA cloning of a receptor for tumor necrosis factor and demonstration of a shed form of the receptor", Proc. Natl. Acad. Sci. U.S.A. 87:6151-6155 (1990).
Novick et al., "Soluble cytokine receptors are present in normal human urine", J. Exp. Med. 170:1409-1414 (1989).
Engelmann et al., "Two tum r necr sis factor-binding proteins purified from human urine", J. Bio. Chem. 265:1531-1536 (1990).
Schall et al., "Molecular cloning and expression of a receptor for human tumor necrosis factor", Cell 61:361-370 (1990).
Seckinger et al., "Tumor necrosis factor inhibitor: purification, $NH_3$-terminal amino acid sequence and evidence for anti-inflammatory and immunomodulatory activities", Eur. J. Immunol. 20:1167-1174 (1990).
Hohmann et al., "Expression of the types A and B tumor necrosis factor (TNT) recept rs is independently r gulated, and both receptors mediate activation of the transcription factor NF-kB", J. Bi . Chem. 265:22409-22417 (1990).
Espevik et al., "Characterization of binding and biological effects of monoclonal antibodies against a human tumor necrosis factor receptor", J. Exp. Med. 171:415-426 (1990).
Porteu and Nathan, "Shedding of tumor necrosis factor receptors by activated human neutrophils", J. Exp. Med. 172:599-607 (1990).
Engelmann et al., "Antibodies to a s luble f rm f a tumor necrosis factor (TNF) receptor have TNF-like activity", J. Bio. Chem. 265:14497-14504 (1990).
Seckinger et al., "Characterization of a tumor necrosis factor α (TNF-α) inhibitor: evidence of immunological cross-activity with the TNF receptor", Proc. Natl. Sci. USA 87:5188-5192 (1990).
Gray et al., "Cloning of human tumor necrosis factor (TNF) receptor cDNA and expression of recombinant soluble TNF-binding protein", Proc. Natl. Sci. USA 87:7380-7384 (1990).
Loetscher et al., "Molecular cloning and expression of the human 55 kd tum r necrosis fact r rec ptor", Cell 61:351-359 (1990).
Peppel et al., "Chimaeric TNF-receptor—IgG molecule acts as soluble inhibitor of TNF mediated cytotoxicity", Journal of Cellular Biochem., Abstract, 20th Annual Meetings, Keystone Symposia on Molecular and Cellular Biology, p. 118, Supplement 15F (1991).
Olsson et al., "Isolation and characterization of a tumor necrosis factor binding pr tein fr m urin ", Eur. J. Ha matol. 42:270-275 (1989).
Capon et al., "Desiging CD4 immunoadhesins for AIDS therapy", Nature 337:525-530 (Feb. 9, 1989).
Abstract 92-009794/02 (1992) for EP 464 533.
U.S. Appl. No. 08/444,791—Office Action mailed Jun. 8, 2010.
U.S. Appl. No. 08/444,791—Office Action mailed Oct. 15, 2010.
Declaration of Taruna Arora, PH.D. Under 37 C.F.R. 1.132 with Exhibits A-D, Dec. 16, 2010.
Heller et al., "Amplified Expression of Tumor Necrosis Factor Receptor in Cells Transfected with Epstein-Barr Virus Shuttle Vector cDNA Libraries," J. Biol. Chem. 265(10): 5708-5717 (1990).
Arora et al., "Differences in Binding and Effector Functions Between Classes of TNF Antagonists," Cytokine 45: 124-131 (2009).
Furst et al., "Tumor Necrosis Factor Antagonists: Different Kinetics and/or Mechanisms of Action May Explain Differences in the Risk for Developing Granulomatous Infection," Semin. Arthritis Rheum. 36: 159-167 (2006).
Mitoma et al., "Mechanisms for Cytotoxic Effects of Anti-Tumor Necrosis Factor Agents on Transmembrane Tumor Necrosis Factor α-Expressing Cells," Arthr. & Rheum. 58(5): 1248-1257 (2008).
Strangfeld et al., "Risk of Herpes Zostcr in Patients with Rheumatoid Arthritis Treated with Anti-TNF-α Agents," JAMA 301(7): 737-744 (2009).
Wallis et al., "Granulomatous Infectious Diseases Associated with Tumor Necrosis Factor Antagonists," Clin. Inf. Dis. 38: 1261-1265 (2004).
Wallis et al., "Reactivation of Latent Granulomatous Infections by Infliximab," Clin. Inf. Dis. 41(Suppl 2): S1-S5 (2005).
Winzor et al., "Evaluation of Equilibrium Constants from Precipitin Curves: Interaction of α -Crystallin with an Elicited Monoclonal Antibody," Arch. Biochem. Biophys. 268(1): 221-226 (1989).
Avis, Kenneth, Parental Preparations, Remington Pharmaceutical Sciences, Chapter 85, pp. 1518-1541 (1985).
Center for Drug Evaluation and Research, Guidelines on Sterile Drug Products Produced by Aseptic Processing, Published by the FDA Jun. 1987.
Feldmann et al., "Cytokine production in the rheumatoid joint: implications for treatment," Ann. Rheum. Dis. 49: 480-486 (1990).
Hoogenboom et al., "Construction and Expression of Antibody-Tumor Necrosis Factor Fusion Proteins," Molecular Immunol. 28(9): 1027-1037 (1991).
Smitii, "cDNA Expression: Cloning of the Receptor for Human Tumor Necrosis Factor," Presentation Programme, 29$^{th}$ Midwinter Conference of Immunologists (Jan. 27-30, 1990).
U.S. Appl. No. 08/484,783, filed Jun. 7, 1995, Kohno et al.
U.S. Appl. No. 07/555,274, filed Jul. 19, 1990, Kohno et al.
Final Office action dated Jun. 24, 2011, in U.S. Appl. No. 08/444,791 (Brockhaus, et al.).
U.S. Appl. No. 08/478,995, Lauffler, Leander et al.
Abraham et al., p55 Tumor Necrosis Factor Receptor Fusion Protein in the Treatment of Patients With Severe Sepsis and Septic Shock: JAMA, 19:1531-1538 (1997).
Abraham et al., Lenercept (p55 Tumor Necrosis Factor Receptor Fusion Protein) In Severe Sepsis and Early Septic Shock: A Randomized, Double-Blind, Placebo-Controlled, Multicenter Phase III Trial With 1,342 Patients, Crit Care Med, 29:503-510 (2001).
Aggarwal et al., Characterization of Receptors for Human Tumour Necrosis Factor and Their Regulation by γ-Interferon, Nature, 318:665-667 (1985).
Aggarwal et al., Induction of Receptors for Tumor Necrosis Factor-α by Interferons Is Not a Major Mechanism for Their Synergistic Cytotoxic Response, J. Biol. Chem., 262:10000-10007 (1987).
Aggarwal et al., Human tumour necrosis factors: structure and receptor interactions, in Tumor necrosis factor and related cytotoxins, pp. 39-51, (Ciba Foundation symposium 131), Wiley, Chichester (1987).
Arenzana-Seisdedos et al., Immunoregulatory Mediators in the Pathogenesis of Rheumotoid Arthritis, Scand. J. Rheumatol., Supplement 66:13-17 (1987).
Aruffo et al., Molecular Cloning of a CD28 cDNA by a High-Efficiency COS Cell Expression System, Proc. Natl. Acad. Sci. USA, 84:8573-8577 (1987).
Ashkenazi et al., Protection Against Endotoxic Shock by a Tumor Necrosis Factor Receptor Immunoadhesin, Proc. Natl. Acad. Sci., U.S.A. 88:10535-10539 (1991).
Ayala, Modem Genetics, Benjamin/Cummings, Publ. Co., Menlo Park CA, p. 45, (1980).
Baglioni et al., Binding of Human Tumor Necrosis Factor to High Affinity Receptors on HeLa and Lymphoblastoid Cells Sensitive to Growth Inhibition, J. Biol. Chem., 260:13395-13397 (1985).
Benjamini et al., Antibody Structure, in Immunology: A Short Course, 3rd ed., Wiley-Liss New York, 61-69 (1996).
Branellec et al., TNF: Antitumoral Agent at the Border Lines of Immunity and Inflammation, Path. Biol., 39:230-239 (1991).
Brockhaus et al., Identification of Two Types of Tumor Necrosis Factor Receptors on Human Cell Lines by Monoclonal Antibodies, Proc. Natl. Acad. Sci. USA, 87:3127-3131 (1990).
Carter et al., Purification, Cloning, Expression and Biological Characterization of an Interleukin-1 Receptor Antagonist Protein, Nature, 344:633-638 (1990).
Carpenter et al., Epidermal Growth Factor, J. Biol. Chem., 265:7709-7712 (1990).
Carpenter, Receptors for Epidermal Growth Factor and Other Polypeptide Mitogens, Ann. Rev, Biochem., 56:881-914 (1987).
Casadei et al., Expression and Secretion of Aequorin as a Chimeric Antibody by Means of a Mammalian Expression Vector, Proc. Natl. Acad. Sci., U.S.A. 87:2047-2051 (1990).
Coffman et al., The Role of Helper T Cell Products in Mouse B Cell Differentiation and Isotype Regulation, Immunol. Rev., 102:5-28 (1988).

Creasey et al., A High Molecular Weight Component of the Human Tumor Necrosis Factor Receptor is Associated With Cytotoxicity, Proc. Natl. Acad. Sci. USA, 84:3293-3297 (1987).
Dayer, Chronic Inflammatory Joint Diseases: Natural Inhibitors of Interleukin 1 and Tumor Necrosis Factor α, J. Rheumatol, 18 (Suppl. 27): 71-75 (1991).
Dower et al., Human Cytokine Receptors, J. Clin. Immunol., 10:289-299 (1990).
Eisenberg et al., Primary Structure and Functional Expression From Complementary DNA of a Human Interleukin-1 Receptor Antagonist, Nature, 343:341-346 (1990).
Ellison et al., The Nucleotide Sequence of a Human Immunoglobulin Cγ1 Gene, Nucleic Acids Res. 10(13): 4071-79 (1982).
Esmon, The Roles of Protein C and Thrombomodulin in the Regulation of Blood Coagulation, J. Biol. Chem., 264:4743-4746 (1989).
European Search Report for EP 97 12 0664, dated Mar. 9, 1998.
Fell et al., Genetic Construction and Characterization of a Fusion Protein Consisting of a Chimeric F(ab') With Specificity for Carcinomas and Human IL-2, J. Immunol., 146:2446-2452 (1991).
Fernandez-Botran et al., A Soluble, High-Affinity, Interleukin-4-Binding Protein is Present in the Biological Fluids of Mice, Proc. Natl. Acad. Sci., 87:4202-4206 (1990).
Fernandez-Botran, Soluble Cytokine Receptors: Their Role in Immunoregulation, The FASEB Journal, 5:2567-2574 (1991).
Ferrante et al., Inhibition of Tumour Necrosis Factor Alpha (TNF-α)-Induced Neutrophil Respiratory Burst by a TNF Inhibitor, Immunology, 72:440-442 (1991).
Fisher et al., Cloning and Expression of Human Tissue Factor cDNA, Thrombosis Research, 48:89-99 (1987).
Foley et al., An Inhibitor of the Toxicity of Tumour Nectrosis Factor in the Serum of Patients With Sarcoidosis, Tuberculosis and Crohn's Disease, Clin. Exp. Immunol, 80:395-399 (1990).
Fomsgaard et al., Preliminary Study on Treatment of Septic Shock Patients With Antilipopolysaccharide IgG from Blood Donors, Scand. J. Infect. Dis., 21:697-708 (1989).
Garcia et al., High Sensitivity of Transgenic Mice Expressing Soluble TNFR1 Fusion Protein to Mycobacterial Infections: Synergistic Action of TNF and IFN-γ in the Differentiation of Protective Granulomas, Eur. J. Immunol., 27:3182-3190 (1997).
Gascoigne et al., Secretion of a Chimeric T-Cell Receptor-Immunoglobulin Protein, Proc. Natl. Acad. Sci USA, 84:2936-2940 (1987).
Gehr et al., Both Tumor Necrosis Factor Receptor Types Mediate Proliferative Signals in Human Mononuclear Cell Activation, J. Immunol., 149:911-917 (1992).
Gillies et al., Targeting Human Cytotoxic T Lymphocytes to Kill Heterologous Epidermal Growth Factor Receptor-Bearing Tumor Cells, J. Immunol., 144:1067-1071 (1991).
Goodman, Identification of Antigenic Determinants, in Basic & Clinical Immunol., 24-25 (1982).
Goodman, Immunogenicity & Antigenic Specificity, in Basic & Clinical Immunol., 101-108 (1991).
Goodwin et al., Molecular cloning and Expression of the Type 1 and Type 2 Murine Receptors for Tumor Necrosis Factor, Molecular and Cellular Biology, 11:3020-3026 (1991).
Gray et al, Cloning and Expression of cDNA for Human Lymphotoxin, a Lymphokine With Tumour Necrosis Activity, Nature, 312:721-724 (1984).
Grundmann et al., Characterization of cDNA Coding for Human Factor XIIIa, Proc. Natl. Acad. Sci. USA, 83:8024-8028 (1986).
Haak-Frendscho et al., Inhibition of TNF by a TNF Receptor Immunoadhesin, J. Immunol., 152:1347-1353 (1994).
Hannum et al., Interleukin-1 Receptor Antagonist Activity of a Human Interleukin-1 Inhibitor, Nature, 343:336-340 (1990).
Heflin et al., Prevention by Granulocyte Depletion of Increased Vascular Permeability of Sheep Lung Following Endotoxemia, J. Clin. Invest., 68:1253-1260 (1981).
Himmler et al., Molecular Cloning and Expression of Human and Rat Tumor Necrosis Factor Receptor Chain (p60) and Its Soluble Derivative, Tumor Necrosis Factor-Binding Protein, DNA and Cell Biology, 9:705-715 (1990).
Hobart, The Immune System: A Course on the Molecular and Cellular Basis of immunity, Blackwell Scientific Pubs, p. 7 (1975).
Holtmann et al., Down Regulation of the Receptors for Tumor Necrosis Factor by Intereukin 1 and 4β-Phorbol-12-Myristate-13-Acetate, J. Immunol., 139:1161-1167 (1987).
Idzerda et al., Human Interleukin 4 Receptor Confers Biological Responsiveness and Defines a Novel Receptor Superfamily, J. Exp. Med., 171:861-873 (1990).
Imamura et al., Expression of Tumor Necrosis Factor Receptors on Human Monocytes and Internalization of Receptor Bound Ligand, J. Immunol., 139:2989-2992 (1987).
Ishikura et al., Differential Biologic Effects Resulting From Bimodal Binding of Recombinant Human Tumor Necrosis Factor to Myeloid Leukemia Cells, Blood, 73:419-424 (1989).
Israel et al., Binding of Human TNF-α to High-Affinity Cell Surface Receptors: Effect of IFN, Immunology Letters, 12:217-224 (1986).
Jacobs et al., Pharmacokinetic Parameters and Biodistribution of Soluble Cytokine Receptors, International Review of Expermental Pathology, 34B:123-135 (1993).
Jones et al, Structure of Tumour Necrosis Factor, Nature, 338:225-228 (1989).
Kaczmarski et al., The Cytokine Receptor Superfamily, Blood Reviews, 5:193-203 (1991).
Kaushansky, Structure-Function Relationships of the Hematopoietic Growth Factors, Proteins: Structure, Function & Genetics, 12:1-9 (1992).
Keegan et al., The Interleukin-4 Receptor. Signal Transduction by a Hematopoietin Receptor, Journal of Leukocyte Biology, 55:272-279 (1994).
Keegan et al., Interleukin 4 Receptor: Signaling Mechanisms, Immunology Today, 15:423-432 (1994).
Kleinau et al., Importance of CD23 for Collagen-Induced Arthritis: Delayed Onset and Reduced Severity in CD23-Deficient Mice, J. Immunol., 162:4266-4270 (1999).
Klinkert et al., TNF-α Receptor Fusion Protein Prevents Experimental Auto-Immune Encephalomyelitis and Demyelination in Lewis Rats: an Overview, The Journal of Neuroimmunology, 72:163-168 (1997).
Kohno et al., A Second Tumor Necrosis Factor Receptor Gene Product Can Shed a Naturally Occurring Tumor Necrosis Factor Inhibitor, Proc. Natl. Acad. Sci. USA, 87:8331-8335 (1990).
Kruse et al., Conversion of Human Interleukin-4 Into a High Affinity Antagonist by a Single Amino Acid Replacement, The EMBO Journal, 11:3237-3244 (1992).
Kull et al., Cellular Receptor for $^{125}$I-Labeled Tumor Necrosis Factor: Specific Binding, Affinity Labeling, and Relationship to Sensitivity, Proc. Natl. Acad. Sci. USA, 82:5756-5760 (1985).
Landolfi, A Chimeric IL-2/Ig Molecule Possesses the Functional Activity of Both Proteins, J. Immunol., 146:915-919 (1991).
Langner et al., Structural and Functional Analysis of a TNF Receptor-Immunoglobulin Fusion Protein, New Advances on Cytokines, 349-354 (1992).
Leberthon et al., Enhanced Tumor Uptake of Macromolecules Induced by a Novel Vasoactive Interleukin 2 Immunoconjugate, Cancer Research, 51:2694-2698 (1991).
Lesslauer et al., Recombinant Soluble Tumor Necrosis Factor Receptor Proteins Protect Mice From Lipopolysaccharide-Induced Lethality, Eur. J. Immunol., 21:2883-2886 (1991).
Liabakk et al., A Rapid and Sensitive Immunoassay for Tumor Necrosis Factor Using Magnetic Monodisperse Polymer Particles, Journal of Immunological Methods, 134:253-259 (1990).
Loetscher et al., Efficacy of a Chimeric TNFR-IgG Fusion Protein to Inhibit TNF Activity in Animal Models of Septic Shock, Endotoxin Research Series, 2:455-462•1993).
Loetscher et al., Two distinct human TNF receptors: purification, molecular cloning and expression, in Tumor Necrosis Factor: Structure-Function Relationship and Clinical Application, (3$^{rd}$ International Conference, 1992.
Maliszewski et al., Cytokine Receptors and B Cell Functions: Recombinant Soluble Receptors Specifically Inhibit IL-1 and IL-4 Induced Cell Activities In Vitro, J. Immunol., 144:3028-3033 (1990).
Mohler et al., Soluble Tumor Necrosis Factor (TNF) Receptors Are Effective Therapeutic Agents in Lethal Endotoxemia and Function Simultaneously as Both TNF Carriers and TNF Antagonists, J. Immunol., 151:1548-1561 (1993).

Mori et al., Attenuation of Collagen-Induced Arthritis in 55-kDa TNF Receptor Type 1 (TNFR1)-IgG1-Treated and TNFR1-Deficient Mice, J. Immunol., 157:3178-3182 (1996).
Morrissey et al., Molecular Cloning of the cDNA for Tissue Factor, the Cellular Receptor for the Initiation of the Coagulation Protease Cascade, Cell: 50:129-135 (1987).
Morrison, In Vitro Antibodies: Strategies for Production and Application, Annu. Rev. Immunol., 10:239-265 (1992).
Mosley et al., The Murine Interleukin-4 Receptor: Molecular Cloning and Characterization of Secreted and Membrane Bound Forms, Cell: 59:335-348 (1989).
Novotny et al., A Soluble, Single-Chain T-Cell Receptor Fragment Endowed With Antigen-Combining Properties, Proc. Natl. Acad. Sci. USA, 88:8646-8650 (1991).
Okayama et al., High-Efficiency Cloning of Full-Length cDNA, Molecular and Cellular Biology, 2:161-170 (1982).
Okayama et al., A cDNA Cloning Vector That Permits Expression of cDNA Inserts in Mammalian Cells, Molecular and Cellular Biology, 3:280-289 (1983).
Old, Tumor Necrosis Factor, 2nd Intl Conference on Tumor Necrosis Factor & Related Cytokines, Napa, CA, 1-30 (1989).
Paborsky et al., Purification of Recombinant Human Tissue Factor, Biochemistry, 28:8072-8077 (1989).
Parrillo, Pathogenetic Mechanisms of Septic Shock, New Eng. J. Med., 328:1471-1477 (1993).
Peetre et al., A Tumor Necrosis Factor Binding Protein is Present in Human Biological Fluids, Eur. J. Haematol. 41:414-419 (1988).
Pennica et al., Human Tumour Necrosis Factor: Precursor Structure, Expression and Homology to Lymphotoxin, Nature, 312:724-729 (1984).
Piguet et al., Evolution of Collagen Arthritis in Mice is Arrested by Treatment With Anti-Tumor Necrosis (TNF) Antibody or a Recombinant Soluble TNF Receptor, Immunology, 77 (4):510-514 (1992).
Redfield'et al., Secondary Structure and Topology of Human Interleukin 4 in Solution, Biochemistry, 30:11029-11035 (1991).
Rubin, Binding Receptor Characters Zako and Expression, and Intracellular Events. 2nd Intl Conference on Tumor Necrosis Factor & Related Cytokines, Napa, CA, 94-96 (1989).
Ruddle et al., An Antibody to Lymphotoxin and Tumor Necrosis Factor Prevents Transfer of Experimental Allergic Encephalomyelitis, J. Exp. Med., 172:1193-1200 (1990).
Rutka et al., The Effects of Human Recombinant Tumor Necrosis Factor on Glioma-Derived Cell Lines: Cellular Proliferation, Cytotoxicity, Morphological and Radioreceptor Studies, Int. J. Cancer., 41:573-582 (1988).
Saxne et al., Detection of Tumor Necrosis Factor α But Not Tumor Necrosis Factor β in Rheumatoid Arthritis Synovial Fluid and Serum, Arthritis & Rheumatism, 31:1041-1045 (1988).
Scallon et al., Functional Comparisons of Different Tumour Necrosis Factor Receptor/IgG Fusion Proteins, Cytokine, 7:759-770 (1995).
Scarpati et al., Human Tissue Factor: cDNA Sequence and Chromosome Localization of the Gene, Biochemistry, 26:5234-5238 (1987).
Schleiffenbaum et al., The Tumor Necrosis Factor Receptor and Human Neutrophil Function, J. Clin. Invest., 86:184-195 (1990).
Schnee et al., Construction and Expression of a Recombinant Antibody-Targeted Plasminogen Activator, Proc. Natl. Acad. Sci. USA, 84:6904-6908 (1987).
Seckinger et al., A Human Inhibitor of Tumor Necrosis Factor α, J. Exp. Med. 167:1511-1516 (1988).
Shalaby et al., Receptor Binding and Activation of Polmorphonuclear Neutrophils by Tumor Necrosis Factor-Alpha, Journal of Leukocyte Biology, 41:196-204 (1987).
Shalaby et al., Binding and Regulation of Cellular Function by Monoclonal antibodies Against Human Tumor Necrosis Factor Receptors, J. Exp. Med. 172: 1517-1520 (1990).
Sheehan et al., Generation and Characterization of Hamster Monoclonal Antibodies That Neutralize Murine Tumor Necrosis Factors, Journal of Immunology, 142:3884-3893 (1989).
Shin et al., Expression and Characterization of an Antibody Binding Specificity Joined to Insulin-Like Growth Factor 1: Potential Applications for Cellular Targeting, Proc. Natl. Acad. Sci., 87:5322-5326 (1990).
Sims et al., cDNA Expression Cloning of the IL-1 Receptor, a Member of the Immunoglobulin Superfamily, Science, 241:585-589 (1988).
Sims et al., Cloning the Interleukin 1 Receptor From Human T Cells, Proc. Natl. Acad. Sci., 86:8946-8950 (1989).
Smith et al., The Active Form of Tumor Necrosis Factor is a Trimer, J. Biol. Chem., 262:6951-6954 (1987).
Smith et al., Blocking of HIV-1 Infectivity by a Soluble, Secreted Form of the CD4 Antigen, Science, 238:1704-1707 (1987).
Smith et al., Multimeric Structure of the Tumor Necrosis Factor Receptor of HeLa Cells, J. Biol. Chem., 264:14646-14652 (1989).
Spicer et al., Isolation of cDNA Clones Coding for Human Tissue Factor: Primary Structure of the Protein and cDNA, Proc. Natl. Acad. Sci., 84:5148-5152 (1987).
Staines et al., Collagen Arthritis—What Can It Teach Us?, British Journal of Rheumatology, 33:798-807 (1994).
Strader et al., Structural Basis of β-Adrenergic Receptor Function, The FASEB Journal, 3:1825-1832 (1989).
Suggs et al., Use of Synthetic Oligonucleotides as Hybridization Probes: Isolation of Cloned cDNA Sequences for Human $\beta_2$,-Microglobulin, Proc. Natl. Acad. Sci. U.S.A., 78:6613-6617 (1981).
Tauber et al., Toxicity in Neuronal Cells Caused by Cererospinal Fluid fFom Pneumococcal and Gram-Negative Meningitis, The Journal of Infectious Diseases, 166:1045-1050 (1992).
Thoma et al., Identification of a 60-kD Tumor Necosis Factor (TNF) Receptor as the Major Signal Transducing Component in TNF Responses, J. Exp. Med. 172: 1019-23 (1990).
Tsujimoto et al., Characterization and Affinity Crosslinking of Receptors for Tumor Necrosis Factor on Human Cells, Archives of Biochemistry and Biophysics, 249:563-568 (1986).
Tsujimoto et al, Interferon-γ Enhances Expression of Cellular Receptors for Tumor Necrosis Factor, J. Immunol., 136:2441-2444 (1986).
Tsujimoto et al, Tumor necrosis factor: specific binding and internalization in sensitive and resistant cells, Proc. Natl. Acad. Sci. 82: 7626-30 (1985).
Ulich et al., Intratracheal Administration of Endotoxin and Cytokines, Clinical Immunology & Immunopathology., 72:137-140 (1994).
Unglaub et al., Downregulation of Tumor Necrosis Factor (TNF) Sensitivity Via Modulation of TNF Binding Capacity by Protein Kinase C Activators, J. Exp. Med. 166:1788-1797 (1987).
Van Der Poll et al., Pretreatment with a 55-kDa Tumor Necrosis Factor Receptor-Immunoglobulin Fusion Protein Attenuates Activation of Coagulation, but not of Fibrinolysis, during Lethal Bacteremia in Baboons, The Journal of Infectious Diseases., 176:296-299 (1997).
Van Zee et al., Protection Against Lethal *Escherichia coli* Bacteremia in Baboons (*Papio anubis*) by Pretreatment With a 55-kDa TNF Receptor (CD120a)-Ig Fusion Protein, Ro 45-2081, J. Immunol., 156:2221-2230 (1996).
Wallach et al., Soluble and Cell Surface Receptors for Tumor Necrosis Factor, Progress, Inflammation Research & Therapy, 51-57 (1991).
Wallach et al., Cell surface and soluble TNF receptors, in Tumor Necrosis Factor: Structure-Function Relationship and Clinical Application, (3rd International Conference on Tumor Necrosis Factor and Related Cytokines, Makuhari, Chiba, Nov. 21-25, 1990), Osawa and Bonavida, eds., Basel, Karger, pp. 47-57 (1992).
Wilks, The CD4 Receptor: Post Binding Events, Conformational Change and the Second Site, Molec. Aspects Med., 12:255-265 (1991).
Yamasaki et al., Cloning and Expression of the Human Interleukin-6 (BSF-2/IFNβ 2) Receptor, Science, 241:825-282 (1988).
Yonehara et al., A Cell-Killing Monoclonal Antibody (Anti-Fas) to a Cell Surface Antigen Co-Downregulated With the Receptor of Tumor Necrosis Factor, J. Exp. Med., 169:1747-1765 (1989).
Yoshie et al., Binding and Crosslinking of $^{125}$I-Labeled Recombinant Human Tumor Necrosis Factor to Cell Surface Receptors, J. Biochem., 100: 531-541(1986).
Official Communication relating to an Opposition in EP Application No. 99 100 703.0, Jan. 31, 2006.
H. Engelmann et al., Jour. of Biological Chemistry, vol. 264, Nr. 20, p. 11974-11980, (Jul. 1989).

P.W. Gray et al, Proc. of the Nat'l Acad. of Sci., vol. 87, p. 7380-7884, (Oct. 1990).
H. Engelmann et al., Jour. of Biological Chemistry, vol. 264, p. 531-1536, (Jan. 1990).
H. Loetscher et al,. Cell, vol. 61, p. 351-359 (Apr. 1990).
T.J. Schall et al., Cell, vol. 61, p. 361-370 (Apr. 1990).
R. A. Heller et al., Proc. of the Nat'l Acad. of Sci., vol. 87, pp. 6151-6155, (Aug. 1990).
C. A. Smith et al, Science, vol. 248, pp. 1019-1023, (May 1990).
P. Seckinger et al, Eur. J. Immunology, vol. 20, pp. 1167-1174 (1990).
D. Novick et al, J. Exp. Med., vol. 170, pp. 1409-1414, (1988).
P. Seckinger et al., Jour. of Biological Chem., vol. 264, pp. 22966-11973 (Jul. 1989).
Folks et al., Tumor necrosis factor α induces expression of human immunodeficiency virus in a chronically infected T-cell clone, PNAS, 86:2365-8 (1989).
Lee, Generation of cDNA probes directed by amino acid sequence: cloning of urate oxidase, Science, 239:1288-1291 (1988).
Moreland et al., Treatment of rheumatoid arthritis with a recombinant human tumor necrosis factor receptor (p75)-Fc fusion protein, New England Journal of Medicine, 337:141-147 (1997).
Sandborn et al., Etanercept for active Crohn's disease: a randomized, double-blind, placebo-controlled trial, Gastroenterol., 121:1088-94 (2001).
Wozney, Using purified protein to clone its genes, Methods in Enzymology, 182:738-751 (1990).
Inflammation & Infection: Rheumatoid Arthritis, Drug & Market Development, 67-70 (2003).
Letter to the EPO with new Auxiliary request 3 in appeal proc. against EP 0 471 701, Dec. 31, 2001.
Notification of the Examiner during the prosecution of EP 99100703. 0, Oct. 24, 2000.
Zavoico, Drug & Market Development, 10(7):235-243 (1999).
Heller et al., Abstract at the 2nd International Conference on Tumor Necrosis Factor and Related Cytokines, Napa Valley, California, Jan. 15-20, 1989.
Loetscher et al., Two Distinct Human TNF Receptors: Purification, Molecular Cloning and Expression, 3rd International Conference on Tumor Necrosis Factor and Related Cytokines, Makuhari, Chiba, Japan, Nov. 21-25, 1990.
Inflammation & Infection: Rheumatoid Arthritis, Drug & Market Development, 67-70 (Mar. 2003).
Barone et al., Arthritis Rheum., 42(9)Suppl, Sep. 1999, Abstract 116.
IPS Notice of Opposition to EP 0 939 121 and Statement in Support of the Opposition against EP 0939121, Dec. 30, 2003.
Patentee's Response to Notice of Opposition Request for Oral Hearing, dated Aug. 3, 2004 (with translation).
Answer of the Opponent on the Admissibility of the Oppositions, dated Nov. 3, 2004.
Response to Patentee's Written Submission, dated Jul. 22, 2005.
Opponent's Observations Under R 71a EPC, dated Sep. 22, 2006.
AHP Comments on Oral Hearing and Preliminary Opinion, dated Sep. 22, 2006.
AHP Response to Opponent's Observations, dated Oct. 6, 2006.
US 6,224,867, 05/2001, Smith et al. (withdrawn)

* cited by examiner

Figure 1

```
-185  GAATTCGGGGGGGTTCAAGATCACTGGGACCAGGCCGTGATCTCTATGCCCGAGTCTCAA
-125  CCCTCAACTGTCACCCCAAGGCACTTGGGACGTCCTGGACAGACCGAGTCCCGGGAAGCC
 -65  CCAGCACTGCCGCTGCCACACTGCCCTGAGCCCAAATGGGGGAGTGAGAGGCCATAGCTG
          -28.
 -30        MetGlyLeuSerThrValProAspLeuLeuLeuProLeuValLeuLeuGluLeu
  -5  TCTGGCATGGGCCTCTCCACCGTGCCTGACCTGCTGCTGCCGCTGGTGCTCCTGGAGCTG
                                        ↓1
 -10  LeuValGlyIleTyrProSerGlyValIleGlyLeuValProHisLeuGlyAspArgGlu
  55  TTGGTGGGAATATACCCCTCAGGGGTTATTGGACTGGTCCCTCACCTAGGGGACAGGGAG
                                                  ***
  10  LysArgAspSerValCysProGlnGlyLysTyrIleHisProGlnAsnAsnSerIleCys
 115  AAGAGAGATAGTGTGTGTCCCCAAGGAAAATATATCCACCCTCAAAATAATTCGATTTGC

  30  CysThrLysCysHisLysGlyThrTyrLeuTyrAsnAspCysProGlyProGlyGlnAsp
 175  TGTACCAAGTGCCACAAAGGAACCTACTTGTACAATGACTGTCCAGGCCCGGGGCAGGAT

  50  ThrAspCysArgGluCysGluSerGlySerPheThrAlaSerGluAsnHisLeuArgHis
 235  ACGGACTGCAGGGAGTGTGAGAGCGGCTCCTTCACCGCTTCAGAAAACCACCTCAGACAC

  70  CysLeuSerCysSerLysCysArgLysGluMetGlyGlnValGluIleSerSerCysThr
 295  TGCCTCAGCTGCTCCAAATGCCGAAAGGAAATGGGTCAGGTGGAGATCTCTTCTTGCACA

  90  ValAspArgAspThrValCysGlyCysArgLysAsnGlnTyrArgHisTyrTrpSerGlu
 355  GTGGACCGGGACACCGTGTGTGGCTGCAGGAAGAACCAGTACCGGCATTATTGGAGTGAA
                    ***                 ***
 110  AsnLeuPheGlnCysPheAsnCysSerLeuCysLeuAsnGlyThrValHisLeuSerCys
 415  AACCTTTTCCAGTGCTTCAATTGCAGCCTCTGCCTCAATGGGACCGTGCACCTCTCCTGC

 130  GlnGluLysGlnAsnThrValCysThrCysHisAlaGlyPhePheLeuArgGluAsnGlu
 475  CAGGAGAAACAGAACACCGTGTGCACCTGCCATGCAGGTTTCTTTCTAAGAGAAAACGAG

 150  CysValSerCysSerAsnCysLysLysSerLeuGluCysThrLysLeuCysLeuProGln
 535  TGTGTCTCCTGTAGTAACTGTAAGAAAAGCCTGGAGTGCACGAAGTTGTGCCTACCCCAG

 170  IleGluAsnValLysGlyThrGluAspSerGlyThrThrValLeuLeuProLeuValIle
 595  ATTGAGAATGTTAAGGGCACTGAGGACTCAGGCACCACAGTGCTGTTGCCCCTGGTCATT

 190  PhePheGlyLeuCysLeuLeuSerLeuLeuPheIleGlyLeuMetTyrArgTyrGlnArg
 655  TTCTTTGGTCTTTGCCTTTTATCCCTCCTCTTCATTGGTTTAATGTATCGCTACCAACGG

 210  TrpLysSerLysLeuTyrSerIleValCysGlyLysSerThrProGluLysGluGlyGlu
 715  TGGAAGTCCAAGCTCTACTCCATTGTTTGTGGGAAATCGACACCTGAAAAAGAGGGGGAG
                                        ***
 230  LeuGluGlyThrThrThrLysProLeuAlaProAsnProSerPheSerProThrProGly
 775  CTTGAAGGAACTACTACTAAGCCCCTGGCCCCAAACCCAAGCTTCAGTCCCACTCCAGGC

 250  PheThrProThrLeuGlyPheSerProValProSerSerThrPheThrSerSerSerThr
 835  TTCACCCCCACCCTGGGCTTCAGTCCCGTGCCCAGTTCCACCTTCACCTCCAGCTCCACC

 270  TyrThrProGlyAspCysProAsnPheAlaAlaProArgArgGluValAlaProProTyr
 895  TATACCCCCGGTGACTGTCCCAACTTTGCGGCTCCCCGCAGAGAGGTGGCACCACCCTAT

 290  GlnGlyAlaAspProIleLeuAlaThrAlaLeuAlaSerAspProIleProAsnProLeu
 955  CAGGGGGCTGACCCCATCCTTGCGACAGCCCTCGCCTCCGACCCCATCCCCAACCCCCTT
```

Figure 1 (cont.

```
 310  GlnLysTrpGluAspSerAlaHisLysProGlnSerLeuAspThrAspAspProAlaThr
1015  CAGAAGTGGGAGGACAGCGCCCACAAGCCACAGAGCCTAGACACTGATGACCCCGCGACG

 330  LeuTyrAlaValValGluAsnValProProLeuArgTrpLysGluPheValArgArgLeu
1075  CTGTACGCCGTGGTGGAGAACGTGCCCCCGTTGCGCTGGAAGGAATTCGTGCGGCGCCTA

 350  GlyLeuSerAspHisGluIleAspArgLeuGluLeuGlnAsnGlyArgCysLeuArgGlu
1135  GGGCTGAGCGACCACGAGATCGATCGGCTGGAGCTGCAGAACGGGCGCTGCCTGCGCGAG

 370  AlaGlnTyrSerMetLeuAlaThrTrpArgArgArgThrProArgArgGluAlaThrLeu
1195  GCGCAATACAGCATGCTGGCGACCTGGAGGCGGCGCACGCCGCGGCGCGAGGCCACGCTG

 390  GluLeuLeuGlyArgValLeuArgAspMetAspLeuLeuGlyCysLeuGluAspIleGlu
1255  GAGCTGCTGGGACGCGTGCTCCGCGACATGGACCTGCTGGGCTGCCTGGAGGACATCGAG

 410  GluAlaLeuCysGlyProAlaAlaLeuProProAlaProSerLeuLeuArg
1315  GAGGCGCTTTGCGGCCCCGCCGCCCTCCCGCCCGCGCCCAGTCTTCTCAGATGAGGCTGC
1375  GCCCCTGCGGGCAGCTCTAAGGACCGTCCTGCGAGATCGCCTTCCAACCCCACTTTTTTC
1435  TGGAAAGGAGGGGTCCTGCAGGGGCAAGCAGGAGCTAGCAGCCGCCTACTTGGTGCTAAC
1495  CCCTCGATGTACATAGCTTTTCTCAGCTGCCTGCGCGCCGCCGACAGTCAGCGCTGTGCG
1555  CGCGGAGAGAGGTGCGCCGTGGGCTCAAGAGCCTGAGTGGGTGGTTTGCGAGGATGAGGG
1615  ACGCTATGCCTCATGCCCGTTTTGGGTGTCCTCACCAGCAAGGCTGCTCGGGGGCCCCTG
1675  GTTCGTCCCTGAGCCTTTTTCACAGTGCATAAGCAGTTTTTTTTGTTTTTGTTTTGTTTT
1735  GTTTTGTTTTTAAATCAATCATGTTACACTAATAGAAACTTGGCACTCCTGTGCCCTCTG
1795  CCTGGACAAGCACATAGCAAGCTGAACTGTCCTAAGGCAGGGGCGAGCACGGAACAATGG
1855  GGCCTTCAGCTGGAGCTGTGGACTTTTGTACATACACTAAAATTCTGAAGTTAAAAAAAA
1915  AACCCGAATTC
```

Figure 2A

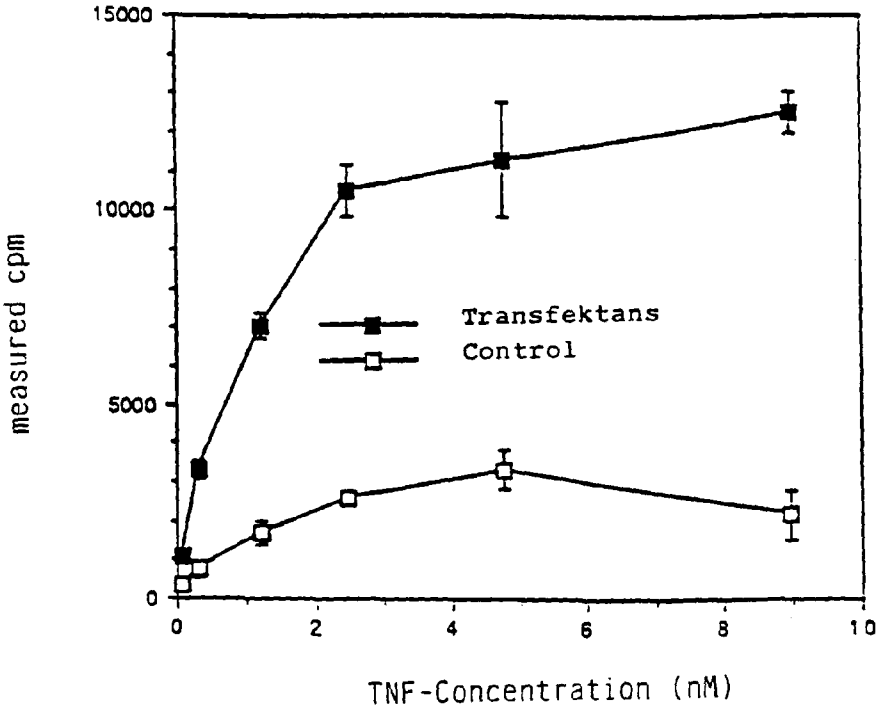



Figure 2B

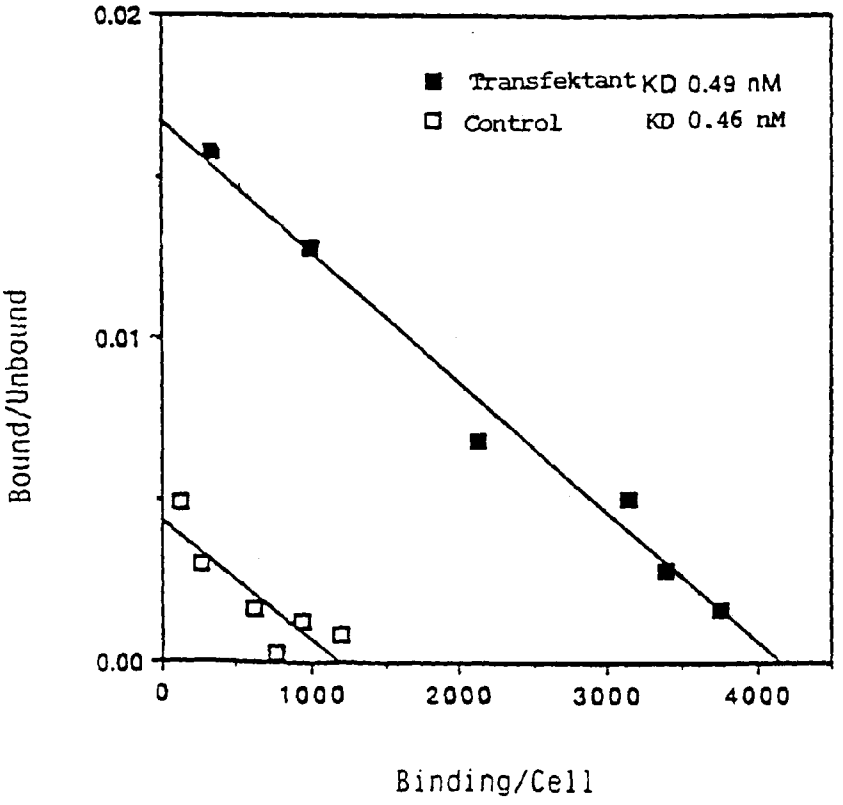


Figure 3

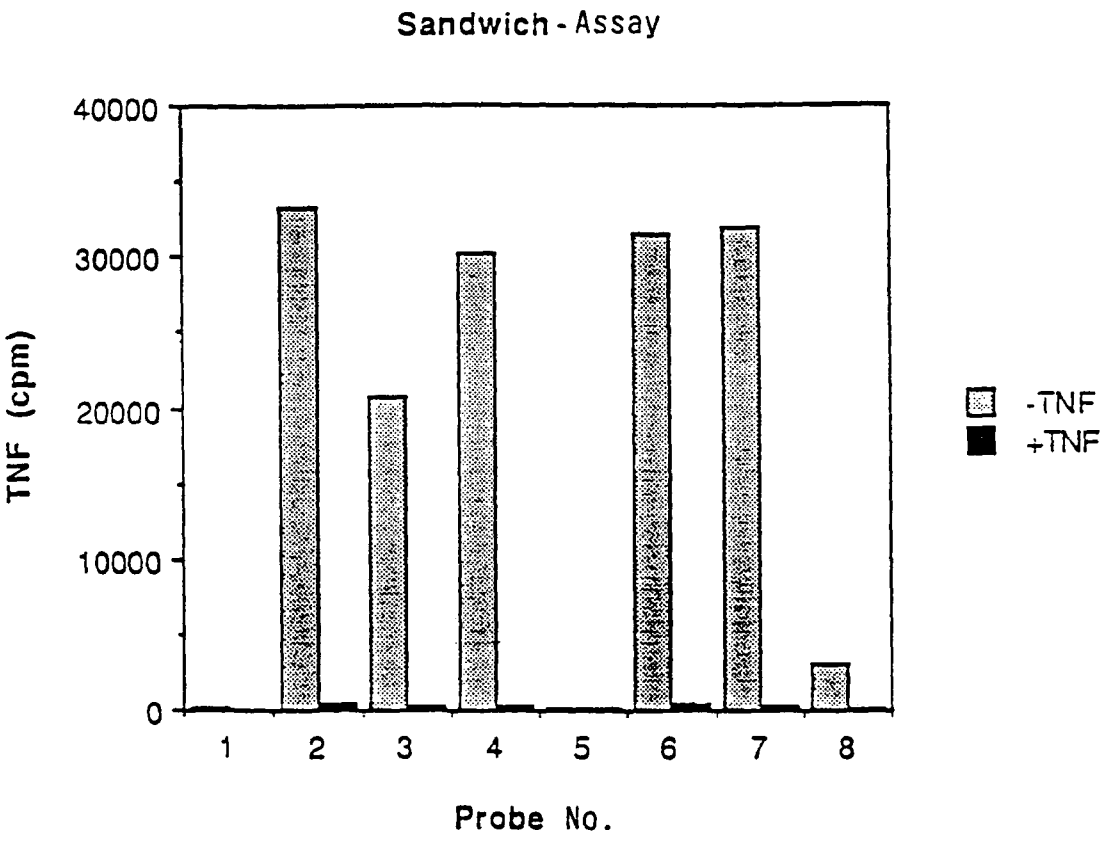

Sandwich - Assay
40000
30000
20000
10000
0
TNF (cpm)
1
2
3
4
5
6
7
8
Probe No.
-TNF
+TNF

Figure 4

```
  1  SerAspSerValCysAspSerCysGluAspSerThrTyrThrGlnLeuTrpAsnTrpVal
  1  TCGGACTCCGTGTGTGACTCCTGTGAGGACAGCACATACACCCAGCTCTGGAACTGGGTT

 21  ProGluCysLeuSerCysGlySerArgCysSerSerAspGlnValGluThrGlnAlaCys
 61  CCCGAGTGCTTGAGCTGTGGCTCCCGCTGTAGCTCTGACCAGGTGGAAACTCAAGCCTGC

 41  ThrArgGluGlnAsnArgIleCysThrCysArgProGlyTrpTyrCysAlaLeuSerLys
121  ACTCGGGAACAGAACCGCATCTGCACCTGCAGGCCCGGCTGGTACTGCGCGCTGAGCAAG

 61  GlnGluGlyCysArgLeuCysAlaProLeuProLysCysArgProGlyPheGlyValAla
181  CAGGAGGGGTGCCGGCTGTGCGCGCCGCTGCCGAAGTGCCGCCCGGGCTTCGGCGTGGCC

 81  ArgProGlyThrGluThrSerAspValValCysLysProCysAlaProGlyThrPheSer
241  AGACCAGGAACTGAAACATCAGACGTGGTGTGCAAGCCCTGTGCCCCGGGGACGTTCTCC

101  AsnThrThrSerSerThrAspIleCysArgProHisGlnIleCysAsnValValAlaIle
301  AACACGACTTCATCCACGGATATTTGCAGGCCCCACCAGATCTGTAACGTGGTGGCCATC

121  ProGlyAsnAlaSerArgAspAlaValCysThrSerThrSerProThrArgSerMetAla
361  CCTGGGAATGCAAGCAGGGATGCAGTCTGCACGTCCACGTCCCCCACCCGGAGTATGGCC

141  ProGlyAlaValHisLeuProGlnProValSerThrArgSerGlnHisThrGlnProSer
421  CCAGGGGCAGTACACTTACCCCAGCCAGTGTCCACACGATCCCAACACACGCAGCCAAGT

161  ProGluProSerThrAlaProSerThrSerPheLeuLeuProMetGlyProSerProPro
481  CCAGAACCCAGCACTGCTCCAAGCACCTCCTTCCTGCTCCCAATGGGCCCCAGCCCCCCA

181  AlaGluGlySerThrGlyAspPheAlaLeuProValGlyLeuIleValGlyValThrAla
541  GCTGAAGGGAGCACTGGCGACTTCGCTCTTCCAGTTGGACTGATTGTGGGTGTGACAGCC

201  LeuGlyLeuLeuIleIleGlyValValAsnCysValIleMetThrGlnValLysLysLys
601  TTGGGTCTACTAATAATAGGAGTGGTGAACTGTGTCATCATGACCCAGGTGAAAAAGAAG

221  ProLeuCysLeuGlnArgGluAlaLysValProHisLeuProAlaAspLysAlaArgGly
661  CCCTTGTGCCTGCAGAGAGAAGCCAAGGTGCCTCACTTGCCTGCCGATAAGGCCCGGGGT

241  ThrGlnGlyProGluGlnGlnHisLeuLeuIleThrAlaProSerSerSerSerSerSer
721  ACACAGGGCCCCGAGCAGCAGCACCTGCTGATCACAGCGCCGAGCTCCAGCAGCAGCTCC

261  LeuGluSerSerAlaSerAlaLeuAspArgArgAlaProThrArgAsnGlnProGlnAla
781  CTGGAGAGCTCGGCCAGTGCGTTGGACAGAAGGGCGCCCACTCGGAACCAGCCACAGGCA
```

Figure 4 (cont.)

```
281   ProGlyValGluAlaSerGlyAlaGlyGluAlaArgAlaSerThrGlySerSerAlaAsp
841   CCAGGCGTGGAGGCCAGTGGGGCCGGGGAGGCCCGGGCCAGCACCGGGAGCTCAGCAGAT

301   SerSerProGlyGlyHisGlyThrGlnValAsnValThrCysIleValAsnValCysSer
901   TCTTCCCCTGGTGGCCATGGGACCCAGGTCAATGTCACCTGCATCGTGAACGTCTGTAGC

321   SerSerAspHisSerSerGlnCysSerSerGlnAlaSerSerThrMetGlyAspThrAsp
961   AGCTCTGACCACAGCTCACAGTGCTCCTCCCAAGCCAGCTCCACAATGGGAGACACAGAT

341   SerSerProSerGluSerProLysAspGluGlnValProPheSerLysGluGluCysAla
1021  TCCAGCCCCTCGGAGTCCCCGAAGGACGAGCAGGTCCCCTTCTCCAAGGAGGAATGTGCC

361   PheArgSerGlnLeuGluThrProGluThrLeuLeuGlySerThrGluGluLysProLeu
1081  TTTCGGTCACAGCTGGAGACGCCAGAGACCCTGCTGGGGAGCACCGAAGAGAAGCCCCTG

381   ProLeuGlyValProAspAlaGlyMetLysProSer
1141  CCCCTTGGAGTGCCTGATGCTGGGATGAAGCCCAGTTAACCAGGCCGGTGTGGGCTGTGT
1201  CGTAGCCAAGGTGGCTGAGCCCTGGCAGGATGACCCTGCGAAGGGGCCCTGGTCCTTCCA
1261  GGCCCCCACCACTAGGACTCTGAGGCTCTTTCTGGGCCAAGTTCCTCTAGTGCCCTCCAC
1321  AGCCGCAGCCTCCCTCTGACCTGCAGGCCAAGAGCAGAGGCAGCGAGTTGTGGAAAGCCT
1381  CTGCTGCCATGGCGTGTCCCTCTCGGAAGGCTGGCTGGGCATGGACGTTCGGGGCATGCT
1441  GGGGCAAGTCCCTGAGTCTCTGTGACCTGCCCCGCCCAGCTGCACCTGCCAGCCTGGCTT
1501  CTGGAGCCCTTGGGTTTTTTGTTTGTTTGTTTGTTTGTTTGTTTGTTTCTCCCCCTGGGC
1561  TCTGCCCAGCTCTGGCTTCCAGAAAACCCCAGCATCCTTTTCTGCAGAGGGGCTTTCTGG
1621  AGAGGAGGGATGCTGCCTGAGTCACCCATGAAGACAGGACAGTGCTTCAGCCTGAGGCTG
1681  AGACTGCGGGATGGTCCTGGGGCTCTGTGCAGGGAGGAGGTGGCAGCCCTGTAGGGAACG
1741  GGGTCCTTCAAGTTAGCTCAGGAGGCTTGGAAAGCATCACCTCAGGCCAGGTGCAGTGGC
1801  TCACGCCTATGATCCCAGCACTTTGGGAGGCTGAGGCGGGTGGATCACCTGAGGTTAGGA
1861  GTTCGAGACCAGCCTGGCCAACATGGTAAAACCCCATCTCTACTAAAAATACAGAAATTA
1921  GCCGGGCGTGGTGGCGGGCACCTATAGTCCCAGCTACTCAGAAGCCTGAGGCTGGGAAAT
1981  CGTTTGAACCCGGGAAGCGGAGGTTGCAGGGAGCCGAGATCACGCCACTGCACTCCAGCC
2041  TGGGCGACAGAGCGAGAGTCTGTCTCAAAAGAAAAAAAAAAAGCACCGCCTCCAAATGCT
2101  AACTTGTCCTTTTGTACCATGGTGTGAAAGTCAGATGCCCAGAGGGCCCAGGCAGGCCAC
2161  CATATTCAGTGCTGTGGCCTGGGCAAGATAACGCACTTCTAACTAGAAATCTGCCAATTT
2221  TTTAAAAAAGTAAGTACCACTCAGGCCAACAAGCCAACGACAAAGCCAAACTCTGCCAGC
2281  CACATCCAACCCCCCACCTGCCATTTGCACCCTCCGCCTTCACTCCGGTGTGCCTGCAG
```

# HUMAN TNF RECEPTOR FUSION PROTEIN

This is a division of application Ser. No. 08/095,640, filed Jul. 21, 1993; now U.S. Pat. No. 5,610,279, which is a continuation application of Ser. No. 07/580,013, filed Sep. 10, 1990, now abandoned. This application claims priority under 35 U.S.C. §119 to application Serial Numbers 3319/89, 746/90 and 1347/90, filed on Sep. 12, 1989, Mar. 8, 1990 and Apr. 20, 1990, respectively, all in Switzerland. This application also claims priority under 35 U.S.C. §119 to European Patent Application Number 90116707.2—(now Patent Number EP 0417563), filed Aug. 31, 1990.

## BACKGROUND OF THE INVENTION

Tumor necrosis factor α (TNFα, also cachectin), discovered as a result of its hemorragic-necrotizing activity on certain tumors, and lymphotoxin (TNFβ) are two closely related peptide factors [3] from the class of lymphokines/cytokines which are both referred to herein-after as TNF [see references 2 and 3]. TNF possesses a broad cellular spectrum of activity. For example, TNF has inhibitory or cytotoxic activity on a series of tumor cell lines [2,3], stimulates the proliferation of fibroblasts and the phagocytic/cytotoxic activity of myeloic cells [4, 5, 6], induces adhesion molecules in endothelial cells or exerts an inhibitory activity on the endothelium [7, 8, 9, 10], inhibits the synthesis of specific enzymes in adipocytes [11] and induces the expression of histo-compatibility antigens [12]. Many of these TNF activities are produced via induction of other factors or by synergistic effects with other factors such as interferons or interleukins [13-16].

TNF is involved in pathological conditions such as shock states in meningococcal sepsis [17], the development of autoimmune glomerulonephritis in mice [18] and cerebral malaria in mice [19] and human beings [41]. The toxic effects of endotoxin appear to be mediated by TNF [20]. Furthermore, TNF can trigger interleukin-1 fever [39]. On the basis of its pleiotropic functional properties, TNF in interaction with other cytokines is involved in additional pathological conditions as a mediator of immune response, inflammation, and other processes.

These biological effects are mediated by TNF via specific receptors. According to present knowledge not only TNFα, but also TNFβ bind to the same receptors [21]. Different cell types differ in their number of TNF receptors [22, 23, 24]. Generally known TNF-binding proteins (TNF-BP) have been detected by covalent bonding to radioactively labelled TNF [24-29], and the following apparent molecular weights of the TNF/TNF-BP complexes obtained have been determined to be: 95/100 kD and 75 kD [24], 95 kD and 75 kD [25], 138 kD, 90 kD, 75 kD and 54 kD [26], 100±5 kD [27], 97 kD and 70 kD [28] and 145 kD [29]. One such TNF/TNF-BP complex was isolated by anti-TNF-antibody immune affinity chromatography and preparative SDS-polyacrylamide gel electrophoreses (SDS-PAGE) [27]. The reductive cleavage of this complex and subsequent SDS-PAGE analysis gave several bands which were not tested for TNF-binding activity. Since the specific conditions which must be used for the cleavage of the complex lead to inactivation of the binding protein [31], the latter has also not been possible. The separation of soluble TNF-BP from human serum or urine by ion exchange chromatography and gel filtration (molecular weight in the region of 50 kD) was described by Olsson et al. [30].

Brockhaus et al. [32] obtained an enriched TNF-BP preparation from membrane extracts of $HL_{60}$ cells by TNFα-ligand affinity chromatography and HPLC which, in turn, was used as an antigen preparation for the production of monoclonal



antibodies against TNF-BP. Using such an immobilized antibody (immune affinity chromatography) Loetscher and Brockhaus obtained an enriched preparation of TNF-BP [31] from an extract of human placenta using TNFα-ligand affinity chromatography and HPLC, which gave a strong broad band at 35 kD, a weak band at about 40 kD and a very weak band in the region between 55 kD and 60 kD on SDS-PAGE analysis. Moreover, the gel showed a protein background smear in the region of 33 kD to 40 kD. The significance of these protein bands was, however, not clear due to the heterogenicity of the starting material which was used (placenta tissue; combined material from several placentas). In the state of the art TNF-BP have already been characterized by a N-terminal partial sequence [European Patent Application, Publication No. 308 378], whereby this sequence differs from the N-terminal partial sequence according to formula (IA) in accordance with the invention. Moreover, the TNF-binding proteins described in the state of the art are soluble, i.e. non-membrane bound, TNF-BP and not membrane-bound, i.e. insoluble, TNF-BP isolated from urine.

## SUMMARY OF THE INVENTION

This invention comprises insoluble, homogenous proteins or soluble or insoluble fragments thereof, capable of binding tumor necrosis factor-(TNF).

This invention also comprises TNF-binding proteins containing amino acid sequences of FIG. **1** or FIG. **4**, proteins containing fragments of these sequences, and proteins analagous to the sequences of FIG. **1** or FIG. **4** or to fragments thereof.

This invention % further comprises DNA sequences encoding the proteins: described above, proteins encoded by these sequences, and antibodies to any of these proteins.

This invention comprises. DNA sequences which combine two partial DNA sequences, one sequence encoding soluble fragments of TNF binding proteins and the other partial sequence encoding all domains except the first domain of the constant region of the heavy chain of human immunoglobulin IgG, IgA, IgM, or IgE, and the recombinant proteins encoded by these sequences.

This invention additionally comprises vectors containing the above DNA sequences, and host systems transfected with such vectors.

This invention finally comprises a process for the isolation of an insoluble homogenous protein capable of binding TNF.

## BRIEF DESCRIPTION OF THE FIGURES

FIG. **1**. Nucleotide sequence (SEQ ID NO: 1) and deduced amino acid sequence (SEQ ID NO: 2) for cDNA clone derived from 55 kD TNF-BP. The 19 amino acid transmembrane region is underlined. Hypothetical glycosylation sites are identified by asterisks.

FIG. **2**. Binding analysis of COS cells transfected with plasmid pN123. Panel 2A—binding of transfected cells to $^{125}$I-TNFα. Panel 2B—Scatchard plot of binding data.

FIG. **3**. Sandwich assays of cells transfected with plasmid pK19. Culture supernatants of cells transfected 30 with pK19 were incubated with anti-55 kD TNF-BP antibody followed by $^{125}$I-TNFα. Columns 1, 5, and 8 are controls. Columns 2, 3, 4, 5, and 6 are five parallel transfections.

FIG. **4**. Nucleotide sequence (SEQ ID NO: 3) and deduced amino acid sequence (SEQ ID NO: 4) for cDNA clones derived from 75/6510 TNF-BP.

## DETAILED DESCRIPTION OF THE INVENTION

The TNF-binding proteins of the present invention are homogenous, insoluble proteins and soluble or insoluble

fragments of such proteins which are capable of binding TNF. These proteins have the ability to bind TNF as measured by standard assays.

The TNF-binding proteins of the present invention include homogenous proteins containing the amino acid sequence depicted in FIG. **1** (SEQ ID NO: 2) or in FIG. **4** (SEQ ID NO: 4), proteins containing fragments of either sequence, and analogues of any such proteins for example proteins containing amino acid sequences analogous to the amino acid sequences of FIG. **1** (SEQ ID NO: 2) or FIG. **4** (SEQ ID NO: 4) or to fragments thereof. An analogue is a protein in which one or more amino acids of the sequences depicted in FIG. **1** (SEQ ID NO: 2) or in FIG. **4** (SEQ ID NO: 4) have had their side-groups chemically modified in a known manner, or those in which one or more amino acids have been replaced or deleted, without thereby eliminating TNF-binding ability. Such analogues may be produced by known methods of peptide chemistry, or by known methods of recombinant DNA technology, such as planned mutagenesis.

The TNF binding activity of the proteins of the present invention may be determined using the assay described in Example 1.

TNF-binding proteins of this invention are obtained as follows:

TNF binding proteins may be isolated from tissues and purified to homogeneity, or isolated from cells which contain membrane-bound TNF binding protein, and purified to homogeneity. One possible method for growing cells and isolating cell extract is described in Example 2, however, other cells types and other growth and isolation methods are well known in the art. Purification of TNF-binding proteins from cell extracts may be performed using the methods described in Examples 4, 5, and 6 in combination with the assay described in Example 1. TNF-binding proteins isolated and purified by these methods were sequenced by well-known methods, as described in Example 7. From these amino acid sequences, DNA probes were produced and used to obtain mRNA encoding TNF binding proteins from which cDNA was made, all by known methods described in Examples 8 and 11. Other well-known methods for producing cDNA are known in the art and may effectively be used. In general, any TNF-binding protein can be isolated from any cell or tissue expressing such proteins using a cDNA probe such as the probe described above, isolating mRNA and transcribing the mRNA into cDNA. Thereafter, the protein can be produced by inserting the cDNA into an expression vector as described in Example 9, such as a virus, plasmid, cosmid, or other vector, inserting the expression vector into a cell, such as the COS cell-described in Example 9 or the insect cell described in Example 10, proliferating the resulting cells, and isolating the expressed TNF-binding protein from the medium or from cell extract as described above. Alternatively, TNF-binding proteins may be chemically synthesized using the sequence described above and an amino acid synthesizer, or manual synthesis using chemical conditions well known to form peptide bonds between selected amino acids. Analogues and fragments of TNF-binding proteins may be produced by the above methods. In the case of analogues, the proteins may be chemically modified, or modified by genetic engineering as described above. These fragments and analogues may then be tested for TNF-binding activity using methods such as the assay of Example 1.

Finally, monoclonal antibodies directed against TNF-binding proteins, such as the antibodies described in Example 3, may be produced by known techniques, and used to isolate TNF-binding proteins.

In more detail, the proteins of the present invention are non-soluble proteins, i.e. for example membrane proteins or so-called receptors, and soluble or non-soluble, fragments thereof, which bind TNF (TNF-BP), in homogeneous form, as well as their physiologically compatible salts. Preferred proteins are those which according to SDS-PAGE under non-reducing conditions are characterized by apparent molecular weights of about 55 kD, 51 kD, 38 kD, 36 kD and 34 kD or 75 kD and 65 kD, especially those with about 55 kD and 75 kD. Furthermore, there are preferred those proteins which are characterized by containing at least one of the following amino acid partial sequences:

(IA) Leu-Val-Pro-His-Leu-Gly-Asp-Arg-Glu-Lys-Arg-Asp-Ser-Val-Cys-Pro-Gln-Gly-Lys-Tyr-Ile-His-Pro-Gln-X-Asn-Ser-Ile (SEQ ID NO: 5)

(IB) Ser-Thr-Pro-Glu-Lys-Glu-Gly-Glu-Leu-Glu-Gly-Thr-Thr-Thr-Lys (SEQ ID NO: 6)

(IIA) Ser-Gln-Leu-Glu-Thr-Pro-Glu-Thr-Leu-Leu-Gly-Ser-Thr-Glu-Glu-Lys-Pro-Leu (SEQ ID NO: 7)

(IIB) Val-Phe-Cys-Thr (SEQ ID NO: 8)

(IIC) Asn-Gln-Pro-Gln-Ala-Pro-Gly-Val-Glu-Ala-Ser-Gly-Ala-Gly-Glu-Ala (SEQ ID NO: 9)

(IID) Leu-pro-Ala-Gln-Val-Ala-Phe-X-Pro-Tyr-Ala-Pro-Glu-Pro-Gly-Ser-Thr-Cys (SEQ ID NO: 10)

(IIE) Ile-X-Pro-Gly-Phe-Gly-Val-Ala-Tyr-Pro-Ala-Leu-Glu (SEQ ID NO: 11)

(IIF) Leu-Cys-Ala-Pro (SEQ ID NO: 12)

(IIG) Val-Pro-His-Leu-Pro-Ala-Asp (SEQ ID NO: 13)

(IIH) Gly-Ser-Gln-Gly-Pro-Glu-Gln-Gln-X-X-Leu-Ile-X-Ala-Pro (SEQ ID NO: 14)

in which X stands for an amino acid residue which could not be unequivocally determined.

A process for the isolation of the TNF-BP in accordance with the invention is also an object of the present invention. This process comprises carrying out essentially the following purification steps in sequence: production of a cell or tissue extract, immune affinity chromatography and/or single or multiple ligand affinity chromatography, high resolution liquid chromatography (HPLC) and preparative SDS-polyacrylamide gel electro phoresis (SDS-PAGE). The combination of the individual purification steps, which are known from the state of the art, is essential to the success of the process in accordance with the invention, whereby individual steps have been modified and improved having regard to the problem to be solved. Thus, for example, the original combined immune affinity chromatography/TNFα-ligand affinity chromatography step originally used for the enrichment of TNF-BP from human placenta [31] has been altered by using a BSA-Sepharose 4B pre-column. For the application of the cell or membrane extract, this pre-column was connected in series with the immune affinity column followed by the ligand affinity column. After the application of the extract the two aforementioned columns were coupled, each eluted and the TNF-BP-active fractions were purified again via a ligand affinity column. The use of a detergent-containing solvent mixture for the performance of the reversed-phase HPLC step is essential to the invention.

Further, an industrial process for the production of high cell densities of mammalian cells from which TNF-BP can be isolated is also an object of the present invention. Such a process comprises using a medium, which has been developed for the specific growth requirements of the cell line used, in combination with a perfusion apparatus as described e.g. in detail in Example 2. By means of such a process there can be produced, for example, in the case of HL-60 cells up to more than 20-fold higher cell densities than usual.

In addition thereto, the present invention is also concerned with DNA sequences coding for proteins and soluble or non-soluble fragments thereof, which bind TNF. Thereunder there are to be understood; for example, DNA sequences coding for non-soluble proteins or soluble as well as non-soluble fragments thereof, which bind TNF, such DNA sequences being selected from the following:

(a) DNA sequences as given FIG. **1** or FIG. **4** as well as their complementary strands, or those which include these sequences;

(b) DNA sequences which hybridize with sequences defined under (a) or fragments thereof;

(c) DNA sequences which, because of the degeneracy of the genetic code, do not hybridize with sequences as defined under (a) and (b), but which code for polypeptides, having exactly the same amino acid sequence.

That is to say, the present invention embraces not only allelic variants, but also those DNA sequences which result from deletions, substitutions and additions from one or more nucleotides of the sequences given in FIG. **1** or FIG. **4**, whereby in the case of the proteins coded thereby there come into consideration, just as before, TNF-BP. One sequence which results from such a deletion is described, for example, in Science 248, 1019-1023, (1990).

There are preferred first of all those DNA sequences which code for such a protein having an apparent molecular weight of about 55 kD, whereby the sequence given in FIG. **1** is especially preferred, and sequences which code for non-soluble as well as soluble fragments of such proteins. A DNA sequence which codes, for example, for such a non-soluble protein fragment extends from nucleotide −185 to 1122 of the sequence given in FIG. **1**. DNA sequences which code for soluble protein fragments are, for example, those which extend from nucleotide −185 to 633 or from nucleotide −14 to 633 of the sequence given in FIG. **1**. There are also preferred DNA sequences which code for a protein of about 75/65 kD, whereby those which contain the partial cDNA sequences shown in FIG. **4** are preferred. Especially preferred DNA sequences in this case are the sequences of the open reading frame of nucleotide 2 to 1,177. The peptides IIA, IIC, IIE, IIF, IIG and IIH are coded by the partial cDNA sequence in FIG. **4**, whereby the insignificant deviations in the experimentally determined amino acid sequences are based on the cDNA-derived sequence with highest probability from the limited resolution of the gas phase sequencing. DNA sequences which code for insoluble (deposited on Oct. 17, 2006 with the American Type Culture Collection under Accession No. PTA 7942) as well as soluble fractions of TNF-binding proteins having an apparent molecular weight of 65 kD/75 kD are also preferred. DNA sequences for such soluble fragments can be determined on the basis of the amino acid sequences derived from the nucleic acid sequences coding for such non-soluble TNF-BP.

The invention is also concerned with DNA sequences which comprise a combination of two partial DNA sequences, with one of the partial sequences coding for those soluble fragments of non-soluble proteins which bind TNF (see above) and the other partial sequence coding for all domains other than the first domain of the constant region of the heavy chain of human immunoglobulins such as IgG, IgA, IgM or IgE, in particular $IgG_1$ or $IgG_3$ subtypes.

The present invention is also concerned with the recombinant proteins coded by any of DNA sequences described above. Of course, there are thereby also included such proteins in whose amino acid sequences amino acids have been exchanged, for example by planned mutagenesis, so that the activity of the TNF-BP or fragments thereof, namely the binding of TNF or the interaction with other membrane components participating in the signal transfer, have been altered or maintained in a desirable manner. Amino acid exchanges in proteins and peptides which do not generally alter the activity of such molecules are known in the state of the art and are described, for example, by H. Neurath and R. L. Hill in "The'Proteins" (Academic Press, New York, 1979, see especially FIG. **6**, page 14). The most commonly occurring exchanges are: Ala/Ser, Val/Ile, Asp/Glu, Thr/Ser, Ala/Gly, Ala/Thr, Ser/Asn, Ala/Val, Ser/Gly, Tyr/Phe, Ala/Pro, Lys/Arg, Asp/Asn, Leu/Ile, Leu/Val, Ala/Glu, Asp/Gly as well as these in reverse. The present invention is also concerned with vectors which contain any of the DNA sequences described above in accordance with the invention and which are suitable for the transformation of suitable pro- and eukaryotic host systems, whereby there are preferred those vectors whose use leads to the expression of the proteins which are coded by any of the DNA sequences described above in accordance with the invention. Finally, the present invention is also concerned with pro- and eukaryotic host systems transformed with such vectors, as well as a process for the production of recombinant compounds in accordance with the invention by cultivating such host systems and subsequently isolating these compounds from the host systems themselves or their culture supernatants.

An object of the present invention are also pharmaceutical preparations which contain at least one of these TNF-BPs or fragments thereof, if desired in combination with other pharmaceutically active substances and/or non-toxic, inert, therapeutically compatible carrier materials.

Finally, the present invention is concerned with the use of such a TNF-BP on the one hand for the production of pharmaceutical preparations and on the other hand for the treatment of illnesses, preferably those in which TNF is involved in their course.

Starting materials for the TNF-BP in accordance with the invention are quite generally cells which contain such TNF-BP [in membrane-bound form] and which are generally accessible without restrictions to a person skilled in the art, such as, for example, HL60 [ATCC No. CCL 240], U 937 [ATCC No. CRL 1593], SW 480 [ATCC No. CCL 228] and HEp2 cells [ATCC No. CCL 23]. These cells can be cultivated according to known methods of the state of the art [40] or, in order to produce high cell densities, according to the procedure already described generally and described in detail in. Example 2 for HL60 cells. TNF-BP can then be extracted from the cells, which are centrifuged-off from the medium and washed, according to known methods of the state of the art using suitable detergents, for example Triton X-114, 1-0-n-octyl-β-D-glucopyranoside (octylglucoside) or 3-[(3-cholylamido-propyl)-dimethylammonio]-1-propane sulphonate (CHAPS), especially using Triton X-100. For the detection of such TNF-BP there can be Used the usually used detection methods for TNF-BP, for example a polyethylene glycol-1-induced precipitation of the $^{125}$I-TNF/TNF-BP complex [27], especially filter-binding tests with radioactively labelled TNF according to Example 1. In order to produce the TNF-BP in accordance with the invention, the general methods of the state of the art used for the purification of proteins, especially of membrane proteins, such as, for example, ion exchange chromatography, gel filtration, affinity chromatography, HPLC and SDS-PAGE can be used. Especially preferred methods for the production of TNF-BP in accordance with the invention are affinity chromatography, especially with TNF-a as the ligand bound to the solid phase, and immune-affinity chromatography, HPLC and SDS-PAGE. The elution of TNF-BP bands which are separated

using SDS-PAGE can be effected according to known methods of protein chemistry, for example using electroelution according to Hunkapiller et al. [34], whereby according to present knowledge the electro-dialysis times given there generally have to be doubled. Thereafter, traces of SDS which still remain can then be removed in accordance with Bosserhoff et al. [50].

The thus-purified TNF-BP can be characterized by methods of peptide chemistry which are known in the state of the art, such as, for example, N-terminal amino acid sequencing of enzymatic well as chemical peptide cleavage. Fragments obtained by enzymatic or chemical cleavage can be separated according to usual methods such as, for example, HPLC and can themselves be subjected to further N-terminal sequencing. Such fragments which themselves bind TNF can be identified using the afore-mentioned detection methods for TNF-BP and are likewise objects of the present invention.

Starting from the thus-obtained amino acid sequence information or the DNA and amino acid sequences given in FIG. **1** as well as in FIG. **4** there can be produced, taking into consideration the degeneracy of the genetic code, according to methods known in the state of the art suitable oligonucleotides [51]. By means of these, again according to known methods of molecular biology [42,43], cDNA or genomic DNA banks can be searched for clones which contain nucleic acid sequences coding for TNF-BP. More-over, using the polymerase chain reaction (PCR) [49] cDNA fragments can be cloned by completely degenerating the amino acid sequence of two spaced apart relatively short segments while taking into consideration the genetic code and introducing into their complementarity suitable oligo-nucleotides as a "primer", whereby the fragment lying between these two sequences can be amplified and identified. The determination of the nucleotide sequence of a such a fragment permits an independent determination of the amino acid sequence of the protein fragment for which it codes. The cDNA fragments obtainable by PCR can also, as already described for the oligonucleotides themselves, be used according to known methods to search for clones containing nucleic acid sequences coding for TNF-BP from cDNA or genomic DNA banks. Such nucleic acid sequences can then be sequenced according to known methods [42]. On the basis of the thus-determined sequences and of the already known sequences for certain receptors, those partial sequences which code for soluble TNF-BP fragments can be determined and cut out from the complete sequence using known methods [42].

The complete sequence or such partial sequences can then be integrated using known methods into vectors described in the state of the art for their multiplication and expression in prokaryotes [42]. Suitable prokaryotic host organisms are, for example, gram-negative and gram-positive bacteria such as, for example, *E. coli* strains such as *E. coli* HB101 [ATCC No. 33 694] or *E. coli* W3110 [ATCC No. 27 325] or *B. subtilis* strains.

Furthermore, nucleic acid sequences in accordance with the invention which code for TNF-BP as well as for TNF-BP fragments can be integrated using known methods into suitable vectors for reproduction and expression in eukaryotic host cells, such as, for example, yeast, insect cells and mammalian cells. Expression of such sequences is preferably effected in mammalian and insect cells.

A typical expression vector for mammalian cells contains an efficient promoter element in order to produce a good transcription rate, the DNA sequence to be expressed and signals for an efficient termination and polyadenylation of the transcript. Additional elements which can be used are "enhancers" which lead to again intensified transcription and sequences which e.g. can bring about a longer half life of the mRNA. For the expression of nucleic acid sequences in which the endogenous sequence fragment coding for a signal peptide is missing, there can be used vectors which contain such suitable sequences which code for signal peptides of other known proteins. See, for example, the vector pLJ268 described by Cullen, B. R. in Cell 46, 973-982 (1986) as well as Sharma, S. et al. in "Current Communications in Molecular Biology", edt. by Gething, M. J., Cold Spring Harbor Lab. (1985), pages 73-78.

Most of these vectors which are used for a transient expression of a particular DNA sequence in mammalian cells contain the replication source of the SV40 virus. In cells which express the T-antigen of the virus (e.g. COS cells), these vectors are reproduced abundantly. A transient expression is, however, not limited to COS cells. In principle any transfectable mammalian cell line can be used for this purpose. Signals which can bring about a strong transcription are e.g. the early and late promoters of SV40, the promoter and enhancer of the "major immediate-early" gene of HCMV (human cytomegalovirus), the LTR's ("long terminal repeats") of retroviruses such as, for example, RSV, HIV and MMTV. There can, however, also be used signals of cellular genes such as e.g. the promoters of the actin and collagenase genes.

Alternatively, however, stable cell lines which have the specific DNA sequence integrated into the genome (chromosome) can also be obtained. For this, the DNA sequence is cotransfected together with a selectable marker, e.g. neomycin, hygromycin, dihydrofolate reductase (dhfr) or hypoxanthin guanine phosphoribosyl transferase (hgpt). The DNA sequence stably incorporated in the chromosome can also be reproduced abundantly. A suitable selection marker for this is, for example, dihydrofolate reductase (dhfr). Mammalian cells (e.g. CHO cells), which contain no intact dhfr gene, are thereby incubated with increasing amounts of methotrexate after transinfection has been effected. In this manner cell lines which contain more than a thousand copies of the desired DNA sequence can be obtained.

Mammalian cells which can be used for the expression are e.g. cells of the human cell lines Hela [ATCC CCL2] and 293 [ATCC CRL 1573] as well as 3T3 [ATCC CCL 163] and L cells, e.g. [ATCC CCL 149], (CHO) cells [ATCC CCL 61], BHK [ATCC CCL 10] cells as well as the CV 1 [ATCC CCL 70] and the COS cell lines [ATCC CRL 1650, CRL 1651].

Suitable expression vectors include, for example, vectors such as pBC12MI [ATCC 67 109], pSV2dhfr [ATCC 37 146], pSVL [Pharmacia, Uppsala, Sweden], pRSVcat [ATCC 37 152] and pMSG [Pharmacia, Uppsala, Sweden]. The vectors "pK19" and "pN123" used in Example 9 are especially preferred vectors. These can be isolated according to known methods from *E. coli* strains HB101(pK19) and HB101 (pN123) transformed with them [42]. These *E. coli* strains have been deposited on the 26 Jan. 1990 at the Deutschen Sammlung von Mikroorganismen and Zellkulturen GmbH (DSM) in Braunschweig, FRG, under DSM 5761 for HB101 (pK19) and DMS 5764 for HB101(pN123). For the expression of proteins which consist of a soluble fragment of non-soluble TNF-BP and an immunoglobulin fragment, i.e. all domains except the first of the constant region of the heavy chain, there are especially suitable pSV2-derived vectors as described, for example, by German, C. in "DNA Cloning" [Vol. II., edt. by Glover, D. M., IRL Press, Oxford, 1985]. The vectors pCD4-Hp (DSM 5315, deposited on 21 Apr. 1989), pDC4-Hγ1 (DSM 5314, deposited on 21 Apr. 1989) and pCD4-Hy3 (DSM 5523, deposited on 14 Sep. 1989) which have been deposited at the Deutschen Sammlung von Mikroorganismen and Zellkulturen GmbH (DSM) in Braunsch-

weig, FRG, and which are described in detail in European Patent Application No. 90107393.2 are especially preferred vectors. This European Patent Specification and the equivalent Applications referred to in Example 11 also contain data with respect to the further use of these vectors for the expression of chimeric proteins (see also Example 11) and for the construction of vectors for the expression of such chimeric proteins with other immunoglobulin fragments.

The manner in which these cells are transfected depends on the chosen expression system and vector system. An overview of these methods is to be found e.g. in Pollard et al., "DNA Transformation of Mammalian Cells" in "Methods in Molecular Biology" [Nucleic Acids Vol. 2, 1984, Walker, J. M., ed, Humana, Clifton, N.J. ]. Further methods are to be found in Chen and Okayama ["High-Efficiency Transformation of Mammalian Cells by Plasmid DNA", Molecular and Cell Biology 7, 2745-2752, 1987] and in Feigner [Feigner et al., "Lipofectin: A highly efficient, lipid-mediated. DNA-transfection procedure", Proc. Nat. Acad. Sci. USA 84, 7413-7417, 1987].

The baculovirus expression system, which has already been used successfully for the expression of a series of proteins (for an overview see Luckow and Summers, Bio/Technology 6, 47-55, 1988), can be used for the expression in insect cells. Recombinant proteins can be produced in authentic form or as fusion proteins. The thus-produced proteins can also be modified such as, for example, glycosylated (Smith et al., Proc. Nat. Acad. Sci. USA 82, 8404-8408, 1987). For the production of a recombinant baculovirus which expresses the desired protein there is used a so-called "transfer vector". Under this there is to be understood a plasmid which contains the heterologous DNA sequence under the control of a strong promoter, e.g. that of the polyhedron gene, whereby this is surrounded on both sides by viral sequences. The vectors "pN113", "pN119" and "pN124" used in Example 10 are especially preferred vectors. These can be isolated according to known methods from *E. coli* strains HB101(pN113), HB101(pN119) and HB101(pN124) transformed with them. These *E. coli* strains have been deposited on the 26 Jan. 1990 at the Deutschen Sammlung von Mikroorganismen and Zellkulturen GmbH (DSM) in Braunschweig, FRG, under DSM 5762 for HB101(pN113), DSM 5763 for HB101 (pN119) and DSM 5765 for HB101(pN124). The transfer vector is then transfected into the insect cells together with DNA of the wild type baculovirus. The recombinant viruses which result in the cells by homologous recombination can then be identified and isolated according to known methods. An overview of the baculovirus expression system and the methods used therein is to be found in Luckow and Summers [52].

Expressed TNF-BP as well as its non-soluble or soluble fractions can then be purified from the cell mass or the culture supernatants according to methods of protein chemistry which are known in the state of the art, such as, for example, the procedure already described on pages 5-6.

The TNF-BP obtained in accordance with the invention can also be used as antigens to produce polyclonal and monoclonal antibodies according to known-techniques [44, 45] or according to the procedure described in Example 3. Such antibodies, especially monoclonal antibodies against the 75 kD TNF-BP species, are also an object of the present invention. Those antibodies which are directed against the 75 kD TNF-BP can be used for the isolation of TNF-BP by modifications of the purification procedure described in detail in Examples 4-6 which are familiar to a person skilled in the art.

On the basis of the high binding affinity of TNF-BP in accordance with the invention for TNF ($K_d$ value in the order of $10^{-9}$-$10^{-10}$ M), these or fragments thereof can be used as diagnostics for the detection of TNF in serum or other body fluids according to methods known in the state of the art, for example in solid phase binding tests or in combination with anti-TNF-BP antibodies in so-called "sandwich" tests.

Moreover, TNF-BP in accordance with the invention can be used on the one hand for the purification of TNF and on the other hand for the detection of TNF agonists and TNF antagonists according to procedures which are known in the state of the art.

The TNF-BP in accordance with the invention as well as their physiologically compatible salts, which can be manufactured according to methods which are known in the state of the art, can also be used for the production of pharmaceutical preparations, primarily those for the treatment of illnesses in which TNF is involved in their course. For this purpose, one or more of the said compounds, where desired or required in combination with other pharmaceutically active substances, can be processed in a known manner with the usually used solid or liquid carrier. materials. The dosage of such preparations can be effected having regard to the usual criteria in analogy to already used preparations of similar activity and structure.

Since the invention has been described hereinbefore in general terms, the following Examples are intended to illustrate details of the invention, but they are not intended to limit its scope in any manner.

## Example 1

### Detection of TNF-Binding Proteins

The TNF-BP were detected in a filter test with human radioiodinated $^{125}$I-TNF. TNF (46, 47) was radioactively labelled with Na$^{125}$I (IMS40, Amersham, Amersham, England) and iodo gene (#28600, Pierce Eurochemie, Oud-Beijerland, Netherlands) according to Fraker and Speck [48]. For the detection of the TNF-BP, isolated membranes of the cells or their solubilized, enriched and purified fractions were applied to moist nitrocellulose filter (0.45µ, BioRad, Richmond, Calif., USA). The filters were then blocked in buffer solution with 1% skimmed milk powder and subsequently incubated with $5 \cdot 10^5$ cpm/ml of $^{125}$I-TNFα ($0.3\text{-}1.0 \cdot 10^8$ cpm/µg) in two batches with and without the addition of 5 µg/ml of non-labelled TNFα, washed and dried in the air. The bound radio-activity was detected semiquantitatively by autoradiography or counted in a gamma-counter. The specific $^{125}$I-TNF-α binding was determined after correction for unspecific binding in the presence of unlabelled TNF-α in excess. The specific TNF-binding in the filter test was measured at various TNF concentrations and analyzed according to Scatchard, whereby a $K_d$ value of $\cdot 10^{-9}$-$10^{-10}$ M was determined.

## Example 2

### Cell Extracts of HL-60 Cells

HL60 cells [ATCC No. CCL 240] were cultivated on an experimental laboratory scale in a RPMI 1640 medium [GIBCO catalogue No. 074-01800], which contained 2 g/l $NaHCO_3$ and 5% foetal calf serum, in a 5% $CO_2$ atmosphere, and subsequently centrifuged.

The following procedure was used to produce high cell densities on an industrial scale. The cultivation was carried out in a 75 l Airlift fermenter (Fa. Chemap, Switzerland) with a working volume of 58 l. For this there was used the cassette membrane system "PROSTAK" (Millipore, Switzerland)

with a membrane surface of 0.32 $m^2$(1 cassette) integrated into the external circulation circuit. The culture medium (see Table 1) was pumped around with a Watson-Marlow pump, Type 603U, with 5 l/min. After a steam sterilization of the installation, whereby the "PROSTAK" system was sterilized separately in autoclaves, the fermentation was started with growing HL-60 cells from a 20 l Airlift fermenter (Chemap). The cell cultivation in the inoculation fermenter was effected in a conventional batch process in the medium according to Table 1 and an initial cell titre of $2\times10^5$ cells/ml. After 4 days the HL60 batch was transferred with a titre of $4.9\times10^6$ cells/ml into the 75 l fermenter. The pH value was held at 7.1 and the $pO_2$ value was held at 25% saturation, whereby the oxygen introduction was effected through a microporous frit. After initial batch fermentation, on the 2nd day the perfusion at a cell titre of $4\times10^6$ cells/ml was started with 30 l of medium exchange per day. On the filtrate side of the medium the conditioned medium was removed and replaced by the addition of fresh medium. The added medium was fortified as follows: Primatone from 0.25% to 0.35%, glutamine from 5 mM to 6 mM and glucose from 4 g/l to 6 g/l. The perfusion rate was then increased on the 3rd and 4th day to 72 l of medium/day and on the 5th day to 100 l of medium/day. The fermentation had finished after 120 hours of continuous cultivation. Exponential cell growth up to $40\times10^6$ cells/ml took place under the given fermentation conditions. The duplication time of the cell population was 20-22 hours to $10\times10^6$ cells/ml and then increased to 30-36 hours with increasing cell density. The proportion of living cells lay at 90-95% during the entire fermentation period. The HL-60 batch was then cooled down in the fermenter to about 12° C. and the cells were harvested by centrifugation (Beckman centrifuge [Model J-6B, Rotor JS], 3000 rpm, 10 min., 4° C.).

TABLE 1

HL-60 medium

| Components | Concentrations mg/l |
|---|---|
| $CaCl_2$ (anhydrous) | 112.644 |
| $Ca(NO_3)_2 \cdot 4H_2O$ | 20 |
| $CuSO_4 \cdot 5H_2O$ | $0.498 \cdot 10^{-3}$ |
| $Fe(NO_3)_3 \cdot 9H_2O$ | 0.02 |
| $FeSO_4 \cdot 7H_2O$ | 0.1668 |
| KCl | 336.72 |
| $KNO_3$ | 0.0309 |
| $MgCl_2$ (anhydrous) | 11.444 |
| $MgSO_4$ (anhydrous) | 68.37 |
| NaCl | 5801.8 |
| $Na_2HPO_4$ (anhydrous) | 188.408 |
| $NaH_2PO_4 \cdot H_2O$ | 75 |
| $Na_2SeO_3 \cdot 5H_2O$ | $9.6 \cdot 10^{-3}$ |
| $ZnSO_4 \cdot 7H_2O$ | 0.1726 |
| D-Glucose | 4000 |
| Glutathion (red.) | 0.2 |
| Hepes buffer | 2383.2 |
| Hypoxanthin | 0.954 |
| Linoleic acid | 0.0168 |
| Lipoic acid | 0.042 |
| Phenol Red | 10.24 |
| Putrescine 2HCl | 0.0322 |
| Na pyruvate | 88 |
| Thymidine | 0.146 |
| Biotin | 0.04666 |
| D-Ca pantothenate | 2.546 |
| Choline chloride | 5.792 |
| Folic acid | 2.86 |
| i-Inositol | 11.32 |
| Niacinamide | 2.6 |
| Nicotinamide | 0.0074 |
| para-Aminobenzoic acid | 0.2 |

TABLE 1-continued

HL-60 medium

| Components | Concentrations mg/l |
|---|---|
| Pyridoxal HCl | 2.4124 |
| Pyridoxin HCl | 0.2 |
| Riboflavin | 0.2876 |
| Thiamin HCl | 2.668 |
| Vitamin $B_{12}$ | 0.2782 |
| L-Alanine | 11.78 |
| L-Aspartic acid | 10 |
| L-Asparagine $H_2O$ | 14.362 |
| L-Arginine | 40 |
| L-Arginine HCl | 92.6 |
| L-Aspartate | 33.32 |
| L-Cystine 2HCl | 62.04 |
| L-Cysteine HCl • $H_2O$ | 7.024 |
| L-Glutamic acid | 36.94 |
| L-Glutamine | 730 |
| L-Glycine | 21.5 |
| L-Histidine | 3 |
| L-Histidine HCl • $H_2O$ | 27.392 |
| L-Hydroxypyroline | 4 |
| L-Isoleucine | 73.788 |
| L-Leucine | 75.62 |
| L-Lysine HCl | 102.9 |
| L-Methionine | 21.896 |
| L-Phenylalanine | 43.592 |
| L-Proline | 26.9 |
| L-Serine | 31.3 |
| L-Threonine | 53 |
| L-Tryptophan | 11.008 |
| L-Tyrosine • 2Na | 69.76 |
| L-Valine | 62.74 |
| Penicillin/streptomycin | 100 U/ml |
| Insulin (human) | 5 µg/ml |
| Tranferrin (human) | 15 µg/ml |
| Bovine serum albumin | 67 µg/ml |
| Primatone RL (Sheffield Products, Norwich NY, USA) | 0.25% |
| Pluronic F68 (Serva, Heidelberg, FRG) | 0.01% |
| Foetal calf serum | 0.3-3% |

The centrifugate was washed with isotonic phosphate buffer (PBS; 0.2 g/l KCl, 0.2 g/l $KH_2PO_4$, 8.0 g/l NaCl, 2.16 g/l $Na_2HPO_4.7H_20$), which had been treated with 5% dimethylformamide, 10 mM benzamidine, 100 U/ml aprotinin, 10 µM leupeptin, 1 µM pepstatin, 1 mM o-phenanthroline, 5 mM iodoacetamide, 1 mM phenyl-methylsulphonyl fluoride (referred to hereinafter as PBS-M). The washed cells were extracted at a density of $2.5\cdot10^8$ cells/ml in PBS-M with Triton X-100 (final concentration 1.0%). The cell extract was clarified by centrifugation (15,000×g, 1 hour; 100,000×g, 1 hour).

## Example 3

### Production of Monoclonal (TNF-BP) Antibodies

A centrifugation supernatant from the cultivation of HL60 cells on an experimental laboratory scale, obtained according to Example 2, was diluted with PBS in the ratio 1:10. The diluted supernatant was applied at 4° C. (flow rate: 0.2 ml/min.) to a column which contained 2 ml of Affigel 10 (Bio Rad Catalogue No. 153-6099) to which had been coupled 20 mg of recombinant human TNF-α [Pennica, D. et al. (1984) Nature 312, 724; Shirai, T. et al. (1985) Nature 313, 803; Wang, A. M. et al. (1985) Science 228, 149] according to the recommendations of the manufacturer. The column was washed at 4° C. and a throughflow rate of 1 ml/min firstly with 20 ml of PBS which contained 0.1% Triton X 114 and there-

after with 20 ml of PBS. Thus-1-enriched TNF-BP was eluted at 22° C. and a flow rate of 2 ml/min with 4 ml of 100 mM glycine, pH 2.8, 0.1% decyl-maltoside. The eluate was concentrated to 10 µl in a Centricon 30 unit [Amicon].

10 µl of this eluate were mixed with 20 µl of complete Freund's adjuvant to give an emulsion. 10 µl of the emulsion were injected according to the procedure described by Holmdahl, R. et al. [(1985), J. Immunol. Methods 32, 379] on each of days 0, 7 and 12 into a hind paw of a narcotized Balb/c mouse.

The immunized mice were sacrificed on day 14, the popliteal lymph nodes were removed, minced and suspended by repeated pipetting in Iscove's medium (IMEM, GIBCO Catalogue No. 074-2200) which contained 2 g/l $NaHCO_3$. According to a modified procedure of De St. Groth and Scheidegger [J. Immunol. Methods (1980), 35, 1] $5\times10^7$ cells of the lymph nodes were fused with $5\times10^7$PAI mouse myeloma cells (J. W. Stocker et al., Research Disclosure, 217, May 1982, 155-157) which were in logarithmic growth. The cells were mixed, collected by centrifugation and resuspended in 2 ml of 50% (v/v) polyethylene glycol in IMEM at room temperature by slight shaking and diluted by the slow addition of 10 ml of IMEM during careful shaking for 10 minutes. The cells were collected by centrifugation and resuspended in 200 ml of complete medium [IMEM+20% foetal calf serum, glutamine (2.0 mM), 2-mercaptoethanol (100 µl), 100 µM hypoxanthine, 0.4 µM aminopterine and 16 µM thymidine (HAT)]. The suspension was distributed on 10 tissue 10 culture dishes each containing 96 wells and incubated at 37° C. for 11 days without changing the medium in an atmosphere of 5% $CO_2$ and a relative humidity of 98%.

The antibodies are distinguished by their inhibitory action on the binding of TNF to HL60 cells or by their binding to antigens in the filter test according to Example 1. The following procedure was used to detect the biological activity of anti(TNF-BP) antibodies: $5\times10^6$ HL60 or U937 cells were incubated in complete RPMI 1640 medium together with affinity-purified monoclonal anti-(TNF-BP) antibodies or control antibodies (i.e. those which are not directed against TNF-BP) in a concentration range of 1 ng/ml to 10 µg/ml. After incubation at 37° C. for one hour the cells were collected by centrifugition and washed with 4.5 ml of PBS at 0° C. They were resuspended in 1 ml of complete RPMI 1640 medium (Example 2) which additionally contained 0.1% sodium azide and $^{125}$I-TNFα ($10^6$ cpm/ml) with or without the addition of unlabelled TNFα (see above). The specific radioactivity of the $^{125}$I-TNFα amounted to 700 Ci/mmol. The cells were incubated at 4° C. for 2 hours, collected and washed 4 times at 0° C. with 4.5 ml of PBS which contained 1% BSA and 0.001% Triton X 100 (Fluka). The radioactivity bound to the cells was measured in a γ-scintillation counter. The cell-bound radioactivity of cells which had not been treated with anti-(TNF-BP) antibodies was determined in a comparative experiment (approximately 10 000 cpm/$5\times10^6$ cells).

## Example 4

### Affinity Chromatography

For the further purification, a monoclonal anti-(55 kD TNF-BP) antibody (2.8 mg/ml gel), obtained according to Example 3, TNFα (3.9 mg/ml gel) and bovine serum albumin (BSA, 8.5 mg/ml gel) were each covalently coupled to CNBr-activated Sepharose 4B (Pharmacia, Uppsala, Sweden) according to the directions of the manufacturer. The cell extract obtained according to Example 2 was passed through the thus-prepared columns which were connected in series in the following sequence: BSA-Sepharose pre-column, immune affinity column (anti-(55 kD-TNF-BP) antibody), TNFα-ligand affinity column. After completion of the application the two last-mentioned columns were separated and washed individually with in each case 100 ml of the following buffer solutions: (1) PBS, 1,0% Triton X-100, 10 mM benzamidine, 100 U/ml aprotinin; (2) PBS, 0.1% Triton X-100, 0.5M NaCl, 10 mM ATP, 10 mM benzamidine, 100 U/ml aprotinin; and (3) PBS, 0.1% Triton X-100, 10 mM benzamidine, 100 U/ml aprotinin. Not only the immune affinity column, but also the TNFα-ligand affinity column were then each eluted with 100 mM glycine pH 2.5, 100 mM NaCl, 0.2% decylmaltoside, 10 mM benzamidine, 100 U/ml aprotinin. The fractions of each column which were active in the filter test according to Example 1 were thereafter combined and neutralized with 1M Tris pH 8.0.

The thus-combined TNF-BP active fractions of the immune affinity chromatography on the one hand and of the TNFα-ligand affinity chromatography on the other hand were, for further purification, again applied to in each case one small TNFa-ligand affinity column. Thereafter, these two columns were washed with in each case 40 ml of (1) PBS, 1.0% Triton X-100, 10 mM benzamidine, 100 U/ml aprotinin, (2) PBS, 0.1% Triton X-100, 0.5M NaCl, 10 mM ATP, 10 mM benzamidine, 100 U/ml aprotinin, (3) PBS, 0.1% Triton X-100, (4) 50 mM Tris pH 7.5, 150 mM NaCl, 1.0% NP-40, 1.0% desoxycholate, 0.1% SDS, (5) PBS, 0.2% decyl-maltoside. Subsequently, the columns were eluted with 100 mM glycine pH 2.5, 100 mM NaCl, 0.2% decylmaltoside. Fractions of 0.5 ml from each column were collected and the fractions from each column which were active according to the filter test (Example 1) were combined and concentrated in a Centricon unit (Amicon, molecular weight exclusion 10,000).

## Example 5

### Separation by Means of HPLC

The active fractions obtained according to Example 4 were each applied according to their different source (immune or ligand affinity chromatography) to C1/C8 reversed phase HPLC columns (ProRPC, Pharmacia, 5×20 mm) which had been equilibrated with 0.1% trifluoroacetic acid, 0.1% octylglucoside. The columns were then eluted with a linear acetonitrile gradient (0-80%) in the same buffer at a flow of 0.5 ml/min. Fractions of 1.0 ml were collected from each column and the active fractions from each column were combined (detection according to Example 1).

## Example 6

### Separation by Means of SDS-PAGE

The fractions which were obtained according to Example 5 and which were active according to the filter test (Example 1) were further separated by SDS-PAGE according to [34]. For this purpose, the samples were heated to 95° C. for 3 minutes in SDS sample buffer and subsequently separated electrophoretically on a 12% acrylamide separation gel with a 5% collection gel. The following standard proteins were used as a reference for the determination of the apparent molecular weights on the SDS-PAGE gel: phosphorylase B (97.4 kD), BSA (66.2 kD), ovalbumin (42.7 kD), carboanhydrase (31.0 kD), soya trypsin inhibitor (21.5 kD) and lysozyme (14.4 kD).

Under the mentioned conditions there were obtained for samples which has been obtained according to Example 4 by TNF-α-ligand affinity chromatography of immune affinity chromatography eluates and which had been further separated by HPLC according to Example 5 two bands of 55 kD and 51 kD as well as three weaker bands of 38 kD, 36 kD and 34 kD. These bands were transferred electro-phoretically during 1 hour at 100 V in 25 mM Tris, 192 mM glycine, 20% methanol on to a PVDF membrane (Immobilon, Millipore, Bedford, Mass. USA) in a Mini Trans Blot System (BioRad, Richmond, Calif., USA). Thereafter, the PVDF membrane was either protein-stained with 0.15% Serva-Blue (Serva, Heidelberg, FRG) in methanol/water/glacial acetic acid (50/40/10 parts by volume) or blocked with skimmed milk powder and subsequently incubated with $^{125}$I-TNFα according to the filter test conditions described in Example 1 in order to detect bands having TNF-BP activity. This showed that all bands produced in the protein staining bonded TNFα specifically. In the Western blot according to Towbin et al. [38] all of these bands also bonded the monoclonal anti-55 kD-TNF-BP antibody produced according to Example 3. In this case, a procedure according to that described in Example 1 with Na$^{125}$I radioactively-labelled, affinity-purified (mouse immuno-globulin-Sepharose-4B affinity column) rabbit-anti-mouse-immunoglobulin antibody was used for the autoradiographic detection of this antibody.

Samples which had been obtained according to Example 4 by two-fold TNF-α-ligand affinity chromatography of the throughput of the immune affinity chromatography and which had been further separated by HPLC according to Example 5 showed under the above-specified SDS-PAGE and blot transfer conditions two additional bands of 75 kD and 65 kD, both of which bonded TNF specifically in the filter test (Example 1). In the Western blot according to Towbin et al. (see above) the proteins of these two bands did not react with the anti-(55 kD TNF-BP) antibody produced according to Example 3. They reacted, however, with a monoclonal antibody which had been produced starting from the 75 kD band (anti-75 kD TNF-BP antibody) according to Example 3.

## Example 7

### Amino Acid Sequence Analysis

For the amino acid sequence analysis, the fractions which had been obtained according to Example 5 and which were active according to the filter test (Example 1) were separated using the SDS-PAGE conditions described in Example 6, but now reducing (SDS sample buffer with 125 mM dithiothreitol). The same bands as in Example 6 were found, but because of the reducing conditions of the SDS-PAGE in comparison to Example 6 all showed an about 1-2 kD higher molecular weight. These bands were then transferred according to Example 6 on to PVDF membranes and stained with 0.15% Serva-Blue in methanol/water/glacial acetic acid (50/400/10 parts by volume) for 1 minute, decolorized with methanol/water/glacial acetic acid (45/48/7 parts by volume), rinsed with water, dried in air and thereafter cut out. The conditions given by Hunkapiller [34] were adhered to in all steps in order to avoid N-terminal blocking. Initially, the purified TNF-BP were used unaltered for the amino acid sequencing. In order to obtain additional sequence information, the TNF-BP after reduction and S-carboxymethylation [Jones, B. N. (1986) in "Methods of Protein Micro-characterisation", J. E. Shively, ed., Humana Press, Clifton N.J., 124-125] were cleaved with cyanogen bromide (Tarr, G. E. in "Methods of Protein Microcharacterisation", 165-166, loc. cit.), trypsin and/or protease K and the peptides were separated by HPLC according to known methods of protein chemistry. Thus-prepared samples were then sequenced in an automatic gas phase microsequencing apparatus (Applied Biosystems Model 470A, ABI, Foster City, Calif., USA) with an on-line automatic HPLC PTH amino acid analyzer (Applied Biosystems Model 120, ABI see above) connected to the outlet, whereby the following amino acid sequences were determined:

1. For the 55 kD band (according to non-reducing SDS-PAGE):

Leu-Val-Pro-His-Leu-Gly-Asp-Arg-Glu-Lys-Arg-Asp-Ser-Val-Cys-Pro-Gln-Gly-Lys-Tyr-Ile-His-Pro-Gln-X-Asn-Ser-Ile (SEQ ID NO: 5),

and

Ser-Thr-Pro-Glu-Lys-Glu-Gly-Glu-Leu-Glu-Gly-Thr-Thr-Thr-Lys (SEQ ID NO: 6) in which X stands for an amino acid residue which could not be determined,

2. for the 51 kD and 38 kD bands (according to non-reducing SDS-PAGE):

Leu-Val-Pro-His-Leu-Gly-Asp-Arg-Glu (SEQ ID NO: 15)

3. for the 6510 band (according to non-reducing SDS-PAGE)

In the N-terminal sequencing of the 6510 band two parallel sequences were determined up to the 15th residue without interruption. Since one of the two sequences corresponded to a partial sequence of ubiquitin [36,37], the following sequence was derived for the 65 kD band:

Leu-Pro-Ala-Gln-Val-Ala-Phe-X-Pro-Tyr-Ala-Pro-Glu-Pro-Gly-Ser-Thr-Cys. (SEQ ID NO: 10)

in which X stands for an amino acid residue which could not be determined.

Additional peptide sequences for 75(65)kDa-TNF-BP were determined:

Ile-X-Pro-Gly-Phe-Gly-Val-Ala-Tyr-Pro-Ala-Leu-Glu (SEQ ID NO: 11)

and

Ser-Gln-Leu-Glu-Thr-Pro-Glu-Thr-Leu-Leu-Gly-Ser-Thr-Glu-Glu-Lys-Pro-Leu (SEQ ID NO: 7) and Val-Phe-Cys-Thr (SEQ ID NO: 8)

and

Asn-Gln-Pro-Gln-Ala-Pro-Gly-Val-Glu-Ala-Ser-Gly-Ala-Gly-Glu-Ala (SEQ ID NO: 9) and Leu-Cys-Ala-Pro (SEQ ID NO: 12)

and

Val-Pro-His-Leu-Pro-Ala-Asp (SEQ ID NO: 13)

and

Gly-Ser-Gln-Gly-Pro-Glu-Gln-Gln-X-X-Leu-Ile-X-Ala-Pro (SEQ ID NO: 14), in which X stands for an amino acid residue which could not be determined.

## Example 8

### Determination of Base Sequences of Complementary DNA (cDNA)

Starting from the amino acid sequence according to formula IA there were synthesized having regard to the genetic code for the amino acid residues 2-7 and 17-23 corresponding completely degenerated oligonucleotides in suitable complementarity ("sense" and "antisense" oligonucleotides). Total cellular RNA was isolated from HL60 cells [42,43] and the first cDNA strand was synthesized by oligo-dT priming or by priming with the "antisense" oligonucleotide using a cDNA synthesis kit (RPN 1256, Amersham, Amersham; England) according to the instructions of the manufacturer. This cDNA strand and the two synthesized degenerate "sense" and "antisense" oligonucleotides were used in a polymerase chain reaction (PCR, Perkin Elmer Cetus, Norwalk, Conn., USA

according to the instructions of the manufacturer) to synthesize as a cDNA fragment the base sequence coding for the amino acid residues 8-16 (formula IA). The base sequence of this cDNA fragment accorded to: 5'-AGGGAGAA-GAGAGATAGTGTGTGTCCC-3' (SEQ ID NO: 16). This cDNA fragment was used as a probe in order to identify according to a known procedure a cDNA clone coding for the 55 kD TNF-BP in a Xgt11-cDNA gene bank from human placenta (42, 43). This clone was then cut according to usual methods from the X-vector and cloned in the plasmids pUC18 (Pharmacia, Uppsala, Sweden) and pUC19 (Pharmacia, Uppsala, Sweden) and in the M13 mp 18/M13 mp 19 bacteriophage (Pharmacia, Uppsala, Sweden) (42, 43). The nucleotide sequence of this cDNA clone was determined using a Sequenase kit (U.S. Biochemical, Cleveland, Ohio, USA) according to the details of the manufacturer. The nucleotide sequence and the amino acid sequence derived therefrom for the 55 kD TNF-BP and its signal peptide (amino acid "−28" to amino acid "0") is given in FIG. **1** using the abbreviations for bases such as amino acids usual in the state of the art. From sequence comparisons with other already known receptor protein sequences there can be determined a N-terminal domain containing approximately 180 amino acids and a C-terminal domain containing 220 amino acids which are separated from one another by a transmembrane region of 19 amino acids (underlined in FIG. **1**) which is typical according to the sequence comparisons. Hypothetical glycosylation sites are characterized in FIG. **1** by asterisks above the corresponding amino acid.

Essentially analogous techniques were used to identify 75/65 kD TNF-BP-coding partial cDNA sequences, whereby however, in this case genomic human DNA and completely degenerated 14-mer and 15-mer "sense" and "antisense" oligonucleotides derived from peptide IIA were used in order to produce a primary 26 by cDNA probe in a polymerase chain reaction. This cDNA probe was then used in a HL-60 cDNA library to identify cDNA clones of different lengths. This cDNA library was produced using isolated HL60 RNA and a cDNA cloning kit (Amersham) according to the details of the manufacturer. The sequence of such a cDNA clone is given in FIG. **4**, whereby repeated sequencing lead to the following correction. A threonine coded by "ACC" not "TCC", has to be at position 3 instead of the serine.

## Example 9

## Expression in COS 1 Cells

Vectors starting from the plasmid "pN11" were constructed for the expression in COS cells. The plasmid "pN11" contains the efficient promoter and enhancer of the "major immediate-early" gene of human cytomegalovirus ("HCMV"; Boshart et al., Cell 41, 521-530, 1985). After the promoter there is situated a short DNA sequence which contains several restriction cleavage sites, which are present only once in the plasmid ("polylinker"), inter alia the cleavage sites for HindIII, BalI, BamHI and PvuII (see sequence).

PvuII
5'-AAGCTTGGCCAGGATCCAGCTGACT-GACTGATCGCGAGATC-3' (SEQ ID NO: 17)
3'-TTCGAACCGGTCCTAGGTCGACTGACT-GACTAGCGCTCTAG-5' (SEQ ID NO: 18)

After these cleavage sites there are situated three translation stop codons in all three reading frames. After the polylinker sequence there is situated the 2nd intron and the polyadenylation signal of the preproinsulin gene of the rat (Lomedico et al., Cell 18, 545-558, 1979). The plasmid also contains the replication origin of the SV40 virus and a fragment from pBR322 which confers *E. coli*-bacteria ampicillin resistance and permits the replication of the plasmid in *E. coli*.

For the construction of the expression vector "pN123", this plasmid "pN11" was cleaved the restriction endo-nuclease PvuII and subsequently treated with alkaline phosphatase. The dephosphorylated vector was thereafter isolated from an agarose gel (V1). The 5'-projecting nucleotides of the EcoRI-cleaved 1.3 kb fragment of the 55 kD TNF-BP-cDNA (see Example 8) were filled in using Klenow enzyme. Subsequently, this fragment was isolated from an agarose gel (F1). Thereafter, V1 and F1 were joined together using T4-ligase. *E. coli* HB101 cells were then transformed with this ligation batch according to known methods [42]. By means of restriction analyses and DNA sequencing according to known methods [42] there were identified transformants which had been transformed with a plasmid and which contained the 1.3 kb EcoRI fragment of the 55 kD TNF-BP-cDNA in the correct orientation for expression via the HCMV-promoter. This vector received the designation "pN123".

The following procedure was used for the construction of the vector "pK19". A DNA fragment which contained only the cDNA coding for the extracellular part of the 55 kD TNF-BP (amino acids −28 to 182 according to FIG. **1**) was obtained by PCR technology (Saiki et al., Science 230, 1350-1354, 1985, see also Example 8). The following oligonucleotides were used in order to amplify the cDNA from "pN123" coding for the extracellular part of the 55 kD TNF-BP:

BAMHI 5'-CACAGGGATCCATAGCTGTCTG-GCATGGGCCTCTCCAC-3' (SEQ ID NO: 19)
ASP718
3'-CGTGACTCCTGAGTCCGTGGTGTAT-TATCTCTAGACCA TGGCCC-5' (SEQ ID NO: 20)

By means of these oligonucleotides there were also introduced two stop codons of the translation after amino acid 182. The thus-amplified DNA fragment was cleaved with BamHI and Asp718, the thereby resulting projecting ends were filled in using Klenow enzyme and this fragment was subsequently isolated from an agarose gel (F2). F2 was then ligated with V1 and the entire batch was used for the transformation of *E. coli* HB101, as already described. Transformants which had been transformed with a plasmid containing the DNA fragment in the correct orientation for the expression via the HCMV-promoter were identified by DNA sequencing (see above). The plasmid isolated therefrom received the designation "pK19".

Transfection of the COS cells with the plasmids "pN123" or "pK19" was carried out according to the lipofection method published by Feigner et al. (Proc. Natl. Acad. Sci. USA 84, 7413-7417, 1987). 72 hours after the transfection had been effected the cells transfected with "pN123" were analyzed for binding with $^{125}$I-TNFα according to known methods. The results of the Scatchard analysis [Scatchard, G., Ann. N.Y. Acad. Sci. 51, 660, 1949] of the thus-obtained binding data (FIG. **2**A) is given in FIG. **2**B. The culture supernatants of the cells transfected with "pK19" were investigated in a "sandwich" test. For this purpose, PVC microtitre plates (Dynatech, Arlington, Va., USA) were sensitized with 100 μl/well of a rabbit-anti-mouse immunoglobulin (10 μg/ml PBS). Subsequently, the plates were washed and incubated (3 hours, 20° C.) with an anti-55 kD TNF-BP antibody which had been detected by its antigen binding and isolated according to Example 3, but which did not inhibit the TNF-binding to cells. The plates were then again washed and incubated overnight at 4° C. with 100 μl/well of the culture supernatant (diluted 1:4 with buffer A containing 1% skimmed milk powder: 50 mM Tris/HCl pH 7.4, 140 mM

NaCl, 5 mM EDTA, 0.02% Na azide). The plates were emptied and incubated at 4° C. for 2 hours with buffer A containing $^{125}$I-TNFα ($10^6$ cpm/ml, 100 μl/well) with or without the addition of 2 μg/ml of unlabelled TNF. Thereafter, the plates were washed 4 times with PBS, the individual wells were cut out and measured in a λ-counter. The results of 5 parallel transfections (columns # 2, 3, 4, 6 and 7), of two control transfections
with the pN11 vector (columns # 1, 5) and of a control with HL60 cell lysate (column # 8) are given in FIG. **3**.

## Example 10

### Expression in Insect Cells

The plasmid "pVL941" (Luckow and Summers, 1989, "High Level Expression of Nonfused Foreign Genes with *Autographa california* Nuclear Polyhedrosis virus Expression Vectors", Virology 170, 31-39) was used for the expression in a baculovirus expression system and was modified as follows. The single EcoRI restriction cleavage site in "pVL941" was removed by cleaving the plasmid with EcoRI and the projecting 5'-end was filled in with Klenow enzyme. The plasmid pVL941/E obtained therefrom was digested with BamHI and Asp718 and the vector trunk was subsequently isolated from an agarose gel. This fragment was ligated with a synthetic oligonucleotide of the following sequence:

BamHI EcoRI Asp718
5'-GATCCAGAATTCATAATAG-3' (SEQ ID NO: 21)
3'-GTCTTAAGTATTATCCATG-5' (SEQ ID NO: 22)

*E. coli* HB101 was transformed with the ligation batch and transformants containing a plasmid in which the oligonucleotide had been incorporated correctly were identified by restriction analysis and DNA sequencing according to known methods (see above); this plasmid was named "pNR704". For the construction of the transfer vector "pN113", this plasmid "pNR704" was cleaved with EcoRI, treated with alkaline phosphatase and the thus-1-produced vector trunk (V2) was subsequently isolated from an agarose gel. The 1.3 kb fragment of the 55 kD TNF-BP-cDNA cleaved with EcoRI as above was ligated with fragment V2. Transformants obtained with this ligation batch, which contained a plasmid containing the cDNA insert in the correct orientation for the expression via the polyhedron promoter, were identified (see above). The vector isolated therefrom received the designation "pN113".

The following procedure was used for the construction of the transfer vector "pN119". The 1.3 kb EcoRI/EcoRI fragment of the 55 kD TNF-BP cDNA in the "pUC19" plasmid (see Example 8) was digested with BanI and ligated with the following synthetic oligonucleotide:

BanI Asp718
5'-GCACCACATAATAGAGATCTGGTACCGGGAA-3' (SEQ ID NO: 23)
3'-GTGTATTATCTCTAGACCATGGCCC-5' (SEQ ID NO: 24)

Two stop codons of the translation after amino acid 182 and a cleavage site for the restriction endo-nuclease Asp718 are incorporated with the above adaptor. After carrying out ligation the batch was digested with EcoRI and Asp718 and the partial 55 kD TNF-BP fragment (F3) was isolated. Furthermore, the plasmid "pNR704", likewise cleaved with Asp718 and EcoRI, was ligated with F3 and the ligation batch was transformed into *E. coli HB*101. The identification of the transformants which contained a plasmid in which the partial 55 kD TNF-BP cDNA had been correctly integrated for the expression was effected as already described. The plasmid isolated from these transformants received the name "pN119".

The following procedure was used for the construction of the transfer vector "pN124". The cDNA fragment coding for the extracellular part of the 55 kD TNF-BP, described in Example 9, was amplified with the specified oligo-nucleotides with the aid of PCR technology as described in Example 9. This fragment was cleaved with BamHI and Asp718 and isolated from an agarose gel (F4). The plasmid "pNR704" was also cleaved with BamHI and Asp718 and the vector trunk (V4) was isolated (see above). The fragments V4 and F4 were ligated, *E. coli* HB101 was transformed therewith and the recombinant transfer vector "pN124" was identified and isolated as described.

The following procedure was used for the transfection 10 of the insect cells. 3 μg of the transfer vector "pN113" were transfected with 1 μg of DNA of the *Autographa californica* nuclear polyhedrosisvirus (AcMNPV) (EP 127839) in Sf9 cells (ATCC CRL 1711). Polyhedron-negative viruses were identified and purified from "plaques" [52]. Sf9 cells were again infected with these recombinant viruses as described in [52]. After 3 days in the culture the infected cells were investigated for TNF-binding using $^{125}$I-TNFα. For this purpose, the transfected cells were washed from the cell culture dish with a Pasteur pipette and resuspended at a cell density of $5\times10^6$ cells/ml of culture medium [52] which contained 10 ng/ml of $^{125}$I-TNF-α, not only in the presence of, but also in the absence of 5 μg/ml of non-labelled TNF-α and incubated on ice for 2 hours. Thereafter, the cells were washed with pure culture medium and the cell-bound radio-activity was counted in a γ-counter (see Table 2).

TABLE 2

| Cells | Cell-bound radioactivity per $10^6$ cells |
|---|---|
| Non-infected cells (control) | 60 cpm |
| Infected cells | 1600 ± 330 cpm [1)] |

[1)] Average and standard deviation from 4 experiments

## Example 11

Analogously to the procedure described in Example 9, the cDNA fragment coding for the extracellular region of the 55 kDa TNF-BP was amplified in a polymerase chain reaction, but now using the following oligonucleotides as the primer:
Oligonucleotide 1:
Sst I 5'-TAC GAG CTC GGC CAT AGC TGT CTG GCA TG-3' (SEQ ID NO: 25)
Oligonucleotide 2:
Sst I 5'-ATA GAG CTC TGT GGT GCC TGA GTC CTC AG-3' (SEQ ID NO: 26)

This cDNA fragment was ligated in the pCD4-Hγ3 vector [DSM 5523; European Patent Application No. 90107393.2; Japanese Patent Application No. 108967/90; U.S. Pat. No. 51,077,390] from which the CD4-cDNA had been removed via the SstI restriction cleavage sites. SstI cleavage sites are situated in vector pCD4-Hγ3 not only in front of, but also behind the CD4-partial sequence fragment. The construction was transfixed in J558 myeloma cells (ATCC No. TIB6) by means of protoplast fusion according to Oi et al. (Procd. Natl. Acad. Sci. USA 80, 825-829, 1983). Transfectants were selected by adding 5 μg/ml of mycophenolic acid and 250 g/ml of xanthin (Traunecker et al., Eur. J. Immunol. 16, 851-

854 [1986]) in basic medium (Dulbecco's modified Eagle's Medium, 10% foetal calf serum, $5\times10^{-5}$M 2-mercaptoethanol). The expression product secreted by the transfixed cells could be purified using usual methods of protein chemistry, e.g. TNF-BP-antibody affinity chromatography. Unless not already specifically indicated, standard procedures as described e.g. by Freshney, R. I. in "Culture of Animal Cells", Alan R. Liss, Inc., New York (1983) were used for the cultivation of the cell lines employed, for the cloning, for the selection or for the expansion of the cloned cells.

REFERENCES

1. G. E. Nedwin, S. L. Naylor, A. Y. Sakaguchi, D. Smith, Jarrett-Nedwin, D. Pennica, D. V. Goeddel and P. W. Gray: Nucl. Acids Res. 12, 6361, 1985
2. B. Beutler and A. Cerami: New England J. Med. 316, 379, 1987
3. L. J. Old: Science 230, 630, 1985
4. G. Trinchieri, M. Kobayashi, M. Rosen, R. Loudon, M. Murphy and B. Perussia: J. exp. Med. 164, 1206, 1986
5. J. Vilcek, V. J. Palombella, D. Henriksen-de Stefano, C. Swenson, R. Feinman, M. Hirai and M. Tsujimoto: J. exp. Med. 163, 632, 1986
6. B. J. Sugarman, B. B. Aggarwal, P. E. Hass, I. S. Figari, M. A. Palladino and H. M. Shepard: Science 230, 943, 1985
7. J. R. Gamble, J. M. Harlan, S. J. Klebanoff and M. A. Vadas: Proc. Natl. Acad. Sci. USA 82, 8667, 1985
8. N. Sato, T. Goto, K. Haranaka, N. Satomi, H. Nariuchi, Y. Mano and Y. Sawasaki: J. Natl. Cancer Inst. 76, 1113, 1986
9. A. H. Stolpen, E. C. Guinan, W. Fiers and J. S. Pober: Am. J. Pathol. 123, 16, 1986
10. J. S. Pober, L. A. Lapierre, A. H. Stolpen, T. A. Brock, T. A. Springer, W. Fiers, M. P. Bevilacqua, D. L. Mendrick and M. A Gimbrone: J. Immunol. 138, 3319, 1987
11. M. Kawakami, P. Pekala, M. Lane and A. Cerami: Proc. Natl. Acad. Sci. USA 22, 912, 1982
12. T. Collins, L. A. Lapierre, W. Fiers, J. L. Strominger and J. S Pober: Proc. Natl. Acad. Sci. USA 83, 446, 1986
13. G. H. W. Wong and D. V. Goeddel: Nature 323, 819, 1986
14. J. W. Lowenthal, D. W. Ballard, E. B•hnlein and W. C. Greene: Proc. Natl. Acad. Sci. USA ja, 2331, 1989
15. M. J. Lenardo, C. M. Fan, T. Maniatis and D. Baltimore: Cell 51, 287, 1989
16. A. E. Goldfeld and T. Maniatis: Proc. Natl. Acad. Sci. USA 86, 1490, 1989
17. A. Waage, A. Halsteuren and T. Espevik: Lancet, Febr. 14, 1987, 355,
18. C. O. Jacob and H. O. McDevitt: Nature 331, 356, 1988
19. G. E. Grau, L. F. Fajardo, P. Piguet, B. Allet, P. Lambert and P. Vassalli: Science 237, 1210, 1987
20. B. Beutler, I. W. Milsark and A. C. Cerami: Science 229, 869, 1985
21. B. B. Aggarwal, T. E. Eessalu and P. E. Hass: Nature 318, 665, 1985
22. M. Tsujimoto, Y. K. Yip and J. Vilcek: Proc. Natl. Acad. Sci. USA 82, 7626, 1985
23. C. Baglioni, S. McCandless, J. Tavernier and W. Fiers: J. Biol. Chem. 260, 13395, 1985
24. P. Hohmann, R. Remy, M. Brockhaus and A. P. G. M. van Loon: J. Biol. Chem., im Druck
25. F. C. Kull, S. Jacobs and P. Cuatrecasas: Proc. Natl. Acad. Sci. USA 82, 5756, 1985
26. A. A. Creasy, R. Yamamoto and Ch. R. Vitt: Proc. Natl. Acad. Sci. USA 84, 3293, 1987
27. G. B. Stauber, R. A. Aiyer and B. B. Aggarwal: J. Biol. Chem. 263, 19098, 1988
28. K. Hirano, K. Yamamoto, Y. Kobayashi and T. Osawa: J. Biochem. 105, 120, 1989
29. Y. Niitsu, N. Watanabe, H. Sone, H. Neda, N. Yamauchi, M. Maeda and I. Urushizaki: J. Biol. Resp. Modifiers 7, 276, 1988
30. I. Olsson, A. Grubb, U. Gullberg, M. Lantz, E. Nilsson, C. Peetre and H. Thysell: Abstract, 2nd Intern. Conference on Tumor Necrosis Factor and Related Cytokines, Napa, Calif., 15.-20. January 1989
31. H. R. Loetscher and M. Brockhaus: Abstract, 2nd Intern. Conference on Tumor Necrosis Factor and Related Cytokines, Napa, Calif., 15.-20. Januar 1989
32. M. Brockhaus, H. Loetscher, H.-P. Hohmann and W. Hunziker: Abstract, 2nd Intern. Conference on Tumor Necrosis Factor and Related Cytokines, Napa, Calif., 15.-20. January 1989
33. C. R. Cantor and P. R. Schimmel, in Biophysical Chemistry, W.H. Freeman, ed., San Francisco, 1980, p. 850
34. M. W. Hunkapiller, E. Lujan, F. Ostrander, L. E. Hood: Methods Enzymol. 91, 227, 1983
35. U. K. Lammli: Nature 227, 680, 1970
36. T. St. John, W. M. Gallatin, M. Siegelman. H. T. Smith, V. A. Fried and I. L. Weissman: Science 231, 845, 1986
37. M. Siegelman, M. W. Bond, W. M. Gallatin, T. St. John, H. T. Smith, V. A. Fried and I. L. Weissman: Science 231, 823, 1986
38. H. Towbin, T. Staehelin and J. Gordon: Proc. Natl. Acad. Sci. USA 76, 4350, 1979
39. Dinarello, Ch. A., in Lymphokines, Vol. 14, E. Pick, ed., p. 1, Academic Press, London, 1987
40. D. J. Merchant, R. H. Kahn and W. H. Murphy: Handbook of Cell and Organ Culture, Burgess Publ. Co., Minneapolis, 1969
41. G. E. Grau, T. E. Taylor, M. E. Molyneux, J. J. Wirima, P. Vassalli, M. Hommel and P. Lambert: New Engl. J. Med. 320, 1586, 1989
42. J. Sambrook, E. F. Fritsch and T. Maniatis: Molecular Cloning, A Laboratory Manual, 2nd ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, Cold Spring Harbor Laboratory Press, 1989
43. F. M. Ausubel, R. Brent, R. E. Kingston, D. D. Moore, J. A. Smith, J. G. Seidman and K. Struhl: Current Protocols in Molecular Biology 1987-1988, S. Wiley and Sons, New York, 1987
44. E. Harlow and D. Lane: Antibodies, A Laboratory Manual, Cold Spring Harbor Laboratory Publications, New York, 1988
45. S. Fazekas de St. Groth and D. Scheidegger: J. Immunol. Methods 35, 1, 1980
46. D. Pennica and D. V. Goeddel, in Lymphokines, Vol. 13, D. R. Webb and D. V. Goeddel, eds. p. 163, Academic Press, London, 1987
47. J. Tavernier, L. Franzen, A. Marmenout, J. van der Heyden, R. Muller, M. Ruysschaert, A. van Vliet, R. Banden and W. Fiers, in Lymphokines, Vol. 13, D. R. Webb and D. V. Goeddel, eds., p. 181, Academic Press, London
48. P. J. Fraker and J. C. Speck: Biochem. Biophys. Res. Commun. 80, 849, 1987
49. D. H. Erlich, D. H. Gelfand, R. K. Saiki: Nature 331, 61, 1988
50. Bosserhoff, J. Wallach and R. W. Frank: J. Chromatogr. 473, 71, 1989
51. R. Lathe: J. Mol. Biol. 183, 1, 1985
52. Luckow and Summers, "A Manual of Methods for Baculovirus Vectors and Insect Cell Culture Procedures", Texas Agricultural Experimental Station, Texas A & M University, Bulletin No. 1555, 2nd edition, 1988

SEQUENCE LISTING

<160> NUMBER OF SEQ ID NOS: 26

<210> SEQ ID NO 1
<211> LENGTH: 2111
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 1

gaattcgggg gggttcaaga tcactgggac caggccgtga tctctatgcc cgagtctcaa 60

ccctcaactg tcaccccaag gcacttggga cgtcctggac agaccgagtc ccgggaagcc 120

ccagcactgc cgctgccaca ctgccctgag cccaaatggg ggagtgagag gccatagctg 180

tctggcatgg gcctctccac cgtgcctgac ctgctgctgc cgctggtgct cctggagctg 240

ttggtgggaa tatacccctc aggggttatt ggactggtcc ctcacctagg ggacagggag 300

aagagagata gtgtgtgtcc ccaaggaaaa tatatccacc ctcaaaataa ttcgatttgc 360

tgtaccaagt gccacaaagg aacctacttg tacaatgact gtccaggccc ggggcaggat 420

acggactgca gggagtgtga gagcggctcc ttcaccgctt cagaaaacca cctcagacac 480

tgcctcagct gctccaaatg ccgaaaggaa atgggtcagg tggagatctc ttcttgcaca 540

gtggaccggg acaccgtgtg tggctgcagg aagaaccagt accggcatta ttggagtgaa 600

aacctttcc agtgcttcaa ttgcagcctc tgcctcaatg ggaccgtgca cctctcctgc 660

caggagaaac agaacaccgt gtgcacctgc catgcaggtt tctttctaag agaaaacgag 720

tgtgtctcct gtagtaactg taagaaaagc ctggagtgca cgaagttgtg cctaccccag 780

attgagaatg ttaagggcac tgaggactca ggcaccacag tgctgttgcc cctggtcatt 840

ttctttggtc tttgcctttt atccctcctc ttcattggtt taatgtatcg ctaccaacgg 900

tggaagtcca agctctactc cattgtttgt gggaaatcga cacctgaaaa agaggggggag 960

cttgaaggaa ctactactaa gcccctggcc ccaaacccaa gcttcagtcc cactccaggc 1020

ttcaccccca ccctgggctt cagtcccgtg cccagttcca ccttcacctc cagctccacc 1080

tatacccccg gtgactgtcc caactttgcg gctccccgca gagaggtggc accaccctat 1140

caggggggctg accccatcct tgcgacagcc ctcgcctccg accccatccc caacccccctt 1200

cagaagtggg aggacagcgc ccacaagcca cagagcctag acactgatga ccccgcgacg 1260

ctgtacgccg tggtggagaa cgtgcccccg ttgcgctgga aggaattcgt gcggcgccta 1320

gggctgagcg accacgagat cgatcggctg gagctgcaga acgggcgctg cctgcgcgag 1380

gcgcaataca gcatgctggc gacctggagg cggcgcacgc cgcggcgcga ggccacgctg 1440

gagctgctgg gacgcgtgct ccgcgacatg gacctgctgg gctgcctgga ggacatcgag 1500

gaggcgcttt gcggccccgc cgccctcccg cccgcgccca gtcttctcag atgaggctgc 1560

gcccctgcgg gcagctctaa ggaccgtcct gcgagatcgc cttccaaccc cacttttttc 1620

tggaaaggag gggtcctgca ggggcaagca ggagctagca gccgcctact tggtgctaac 1680

ccctcgatgt acatagcttt tctcagctgc ctgcgcgccg ccgacagtca gcgctgtgcg 1740

cgcggagaga ggtgcgccgt gggctcaaga gcctgagtgg gtggtttgcg aggatgaggg 1800

acgctatgcc tcatgcccgt tttgggtgtc ctcaccagca aggctgctcg ggggcccctg 1860

gttcgtccct gagccttttt cacagtgcat aagcagtttt ttttgttttt gttttgtttt 1920

gttttgtttt taaatcaatc atgttacact aatagaaact tggcactcct gtgccctctg 1980

cctggacaag cacatagcaa gctgaactgt cctaaggcag gggcgagcac ggaacaatgg 2040

-continued

ggccttcagc tggagctgtg gacttttgta catacactaa aattctgaag ttaaaaaaaa 2100

aacccgaatt c 2111

<210> SEQ ID NO 2
<211> LENGTH: 455
<212> TYPE: PRT
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 2

Met Gly Leu Ser Thr Val Pro Asp Leu Leu Leu Pro Leu Val Leu Leu
1 5 10 15

Glu Leu Leu Val Gly Ile Tyr Pro Ser Gly Val Ile Gly Leu Val Pro
20 25 30

His Leu Gly Asp Arg Glu Lys Arg Asp Ser Val Cys Pro Gln Gly Lys
35 40 45

Tyr Ile His Pro Gln Asn Asn Ser Ile Cys Cys Thr Lys Cys His Lys
50 55 60

Gly Thr Tyr Leu Tyr Asn Asp Cys Pro Gly Pro Gly Gln Asp Thr Asp
65 70 75 80

Cys Arg Glu Cys Glu Ser Gly Ser Phe Thr Ala Ser Glu Asn His Leu
85 90 95

Arg His Cys Leu Ser Cys Ser Lys Cys Arg Lys Glu Met Gly Gln Val
100 105 110

Glu Ile Ser Ser Cys Thr Val Asp Arg Asp Thr Val Cys Gly Cys Arg
115 120 125

Lys Asn Gln Tyr Arg His Tyr Trp Ser Glu Asn Leu Phe Gln Cys Phe
130 135 140

Asn Cys Ser Leu Cys Leu Asn Gly Thr Val His Leu Ser Cys Gln Glu
145 150 155 160

Lys Gln Asn Thr Val Cys Thr Cys His Ala Gly Phe Phe Leu Arg Glu
165 170 175

Asn Glu Cys Val Ser Cys Ser Asn Cys Lys Lys Ser Leu Glu Cys Thr
180 185 190

Lys Leu Cys Leu Pro Gln Ile Glu Asn Val Lys Gly Thr Glu Asp Ser
195 200 205

Gly Thr Thr Val Leu Leu Pro Leu Val Ile Phe Phe Gly Leu Cys Leu
210 215 220

Leu Ser Leu Leu Phe Ile Gly Leu Met Tyr Arg Tyr Gln Arg Trp Lys
225 230 235 240

Ser Lys Leu Tyr Ser Ile Val Cys Gly Lys Ser Thr Pro Glu Lys Glu
245 250 255

Gly Glu Leu Glu Gly Thr Thr Thr Lys Pro Leu Ala Pro Asn Pro Ser
260 265 270

Phe Ser Pro Thr Pro Gly Phe Thr Pro Thr Leu Gly Phe Ser Pro Val
275 280 285

Pro Ser Ser Thr Phe Thr Ser Ser Ser Thr Tyr Thr Pro Gly Asp Cys
290 295 300

Pro Asn Phe Ala Ala Pro Arg Arg Glu Val Ala Pro Pro Tyr Gln Gly
305 310 315 320

Ala Asp Pro Ile Leu Ala Thr Ala Leu Ala Ser Asp Pro Ile Pro Asn
325 330 335

Pro Leu Gln Lys Trp Glu Asp Ser Ala His Lys Pro Gln Ser Leu Asp
340 345 350

Thr Asp Asp Pro Ala Thr Leu Tyr Ala Val Val Glu Asn Val Pro Pro
355 360 365

```
Leu Arg Trp Lys Glu Phe Val Arg Arg Leu Gly Leu Ser Asp His Glu
    370                 375                 380

Ile Asp Arg Leu Glu Leu Gln Asn Gly Arg Cys Leu Arg Glu Ala Gln
385                 390                 395                 400

Tyr Ser Met Leu Ala Thr Trp Arg Arg Arg Thr Pro Arg Arg Glu Ala
                405                 410                 415

Thr Leu Glu Leu Leu Gly Arg Val Leu Arg Asp Met Asp Leu Leu Gly
            420                 425                 430

Cys Leu Glu Asp Ile Glu Glu Ala Leu Cys Gly Pro Ala Ala Leu Pro
        435                 440                 445

Pro Ala Pro Ser Leu Leu Arg
    450                 455


<210> SEQ ID NO 3
<211> LENGTH: 2339
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 3

tcggacaccg tgtgtgactc ctgtgaggac agcacataca cccagctctg gaactgggtt     60

cccgagtgct tgagctgtgg ctcccgctgt agctctgacc aggtggaaac tcaagcctgc    120

actcgggaac agaaccgcat ctgcacctgc aggcccggct ggtactgcgc gctgagcaag    180

caggaggggt gccggctgtg cgcgccgctg ccgaagtgcc gcccgggctt cggcgtggcc    240

agaccaggaa ctgaaacatc agacgtggtg tgcaagccct gtgccccggg gacgttctcc    300

aacacgactt catccacgga tatttgcagg ccccaccaga tctgtaacgt ggtggccatc    360

cctgggaatg caagcaggga tgcagtctgc acgtccacgt cccccacccg gagtatggcc    420

ccaggggcag tacacttacc ccagccagtg tccacacgat cccaacacac gcagccaagt    480

ccagaaccca gcactgctcc aagcacctcc ttcctgctcc caatgggccc cagcccccca    540

gctgaaggga gcactggcga cttcgctctt ccagttggac tgattgtggg tgtgacagcc    600

ttgggtctac taataatagg agtggtgaac tgtgtcatca tgacccaggt gaaaaagaag    660

cccttgtgcc tgcagagaga agccaaggtg cctcacttgc ctgccgataa ggcccggggt    720

acacagggcc ccgagcagca gcacctgctg atcacagcgc cgagctccag cagcagctcc    780

ctggagagct cggccagtgc gttggacaga agggcgccca ctcggaacca gccacaggca    840

ccaggcgtgg aggccagtgg ggccggggag gcccgggcca gcaccgggag ctcagcagat    900

tcttcccctg gtggccatgg gacccaggtc aatgtcacct gcatcgtgaa cgtctgtagc    960

agctctgacc acagctcaca gtgctcctcc caagccagct ccacaatggg agacacagat   1020

tccagcccct cggagtcccc gaaggacgag caggtcccct tctccaagga ggaatgtgcc   1080

tttcggtcac agctggagac gccagagacc ctgctgggga gcaccgaaga gaagcccctg   1140

ccccttggag tgcctgatgc tgggatgaag cccagttaac caggccggtg tgggctgtgt   1200

cgtagccaag gtggctgagc cctggcagga tgaccctgcg aaggggccct ggtccttcca   1260

ggcccccacc actaggactc tgaggctctt tctgggccaa gttcctctag tgccctccac   1320

agccgcagcc tccctctgac ctgcaggcca agagcagagg cagcgagttg tggaaagcct   1380

ctgctgccat ggcgtgtccc tctcggaagg ctggctgggc atggacgttc ggggcatgct   1440

ggggcaagtc cctgagtctc tgtgacctgc cccgcccagc tgcacctgcc agcctggctt   1500

ctggagccct tgggtttttt gtttgtttgt ttgtttgttt gtttgtttct ccccctgggc   1560

tctgcccagc tctggcttcc agaaaacccc agcatccttt tctgcagagg ggctttctgg   1620
```

agaggaggga tgctgcctga gtcacccatg aagacaggac agtgcttcag cctgaggctg 1680

agactgcggg atggtcctgg ggctctgtgc agggaggagg tggcagccct gtagggaacg 1740

gggtccttca agttagctca ggaggcttgg aaagcatcac ctcaggccag gtgcagtggc 1800

tcacgcctat gatcccagca ctttgggagg ctgaggcggg tggatcacct gaggttagga 1860

gttcgagacc agcctggcca acatggtaaa accccatctc tactaaaaat acagaaatta 1920

gccgggcgtg gtggcgggca cctatagtcc cagctactca gaagcctgag gctgggaaat 1980

cgtttgaacc cgggaagcgg aggttgcagg gagccgagat cacgccactg cactccagcc 2040

tgggcgacag agcgagagtc tgtctcaaaa gaaaaaaaaa aagcaccgcc tccaaatgct 2100

aacttgtcct tttgtaccat ggtgtgaaag tcagatgccc agagggccca ggcaggccac 2160

catattcagt gctgtggcct gggcaagata acgcacttct aactagaaat ctgccaattt 2220

tttaaaaaag taagtaccac tcaggccaac aagccaacga caaagccaaa ctctgccagc 2280

cacatccaac cccccacctg ccatttgcac cctccgcctt cactccggtg tgcctgcag 2339

<210> SEQ ID NO 4
<211> LENGTH: 392
<212> TYPE: PRT
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 4

Ser Asp Thr Val Cys Asp Ser Cys Glu Asp Ser Thr Tyr Thr Gln Leu
1 5 10 15

Trp Asn Trp Val Pro Glu Cys Leu Ser Cys Gly Ser Arg Cys Ser Ser
20 25 30

Asp Gln Val Glu Thr Gln Ala Cys Thr Arg Glu Gln Asn Arg Ile Cys
35 40 45

Thr Cys Arg Pro Gly Trp Tyr Cys Ala Leu Ser Lys Gln Glu Gly Cys
50 55 60

Arg Leu Cys Ala Pro Leu Pro Lys Cys Arg Pro Gly Phe Gly Val Ala
65 70 75 80

Arg Pro Gly Thr Glu Thr Ser Asp Val Val Cys Lys Pro Cys Ala Pro
85 90 95

Gly Thr Phe Ser Asn Thr Thr Ser Ser Thr Asp Ile Cys Arg Pro His
100 105 110

Gln Ile Cys Asn Val Val Ala Ile Pro Gly Asn Ala Ser Arg Asp Ala
115 120 125

Val Cys Thr Ser Thr Ser Pro Thr Arg Ser Met Ala Pro Gly Ala Val
130 135 140

His Leu Pro Gln Pro Val Ser Thr Arg Ser Gln His Thr Gln Pro Ser
145 150 155 160

Pro Glu Pro Ser Thr Ala Pro Ser Thr Ser Phe Leu Leu Pro Met Gly
165 170 175

Pro Ser Pro Pro Ala Glu Gly Ser Thr Gly Asp Phe Ala Leu Pro Val
180 185 190

Gly Leu Ile Val Gly Val Thr Ala Leu Gly Leu Leu Ile Ile Gly Val
195 200 205

Val Asn Cys Val Ile Met Thr Gln Val Lys Lys Lys Pro Leu Cys Leu
210 215 220

Gln Arg Glu Ala Lys Val Pro His Leu Pro Ala Asp Lys Ala Arg Gly
225 230 235 240

Thr Gln Gly Pro Glu Gln Gln His Leu Leu Ile Thr Ala Pro Ser Ser
245 250 255

```
Ser Ser Ser Ser Leu Glu Ser Ser Ala Ser Ala Leu Asp Arg Arg Ala
            260                 265                 270

Pro Thr Arg Asn Gln Pro Gln Ala Pro Gly Val Glu Ala Ser Gly Ala
        275                 280                 285

Gly Glu Ala Arg Ala Ser Thr Gly Ser Ser Ala Asp Ser Ser Pro Gly
    290                 295                 300

Gly His Gly Thr Gln Val Asn Val Thr Cys Ile Val Asn Val Cys Ser
305                 310                 315                 320

Ser Ser Asp His Ser Ser Gln Cys Ser Ser Gln Ala Ser Ser Thr Met
                325                 330                 335

Gly Asp Thr Asp Ser Ser Pro Ser Glu Ser Pro Lys Asp Glu Gln Val
            340                 345                 350

Pro Phe Ser Lys Glu Glu Cys Ala Phe Arg Ser Gln Leu Glu Thr Pro
        355                 360                 365

Glu Thr Leu Leu Gly Ser Thr Glu Glu Lys Pro Leu Pro Leu Gly Val
    370                 375                 380

Pro Asp Ala Gly Met Lys Pro Ser
385                 390


<210> SEQ ID NO 5
<211> LENGTH: 28
<212> TYPE: PRT
<213> ORGANISM: Artificial sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic peptide
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (25)..(25)
<223> OTHER INFORMATION: Xaa = unknown amino acid

<400> SEQUENCE: 5

Leu Val Pro His Leu Gly Asp Arg Glu Lys Arg Asp Ser Val Cys Pro
1               5                   10                  15

Gln Gly Lys Tyr Ile His Pro Gln Xaa Asn Ser Ile
            20                  25


<210> SEQ ID NO 6
<211> LENGTH: 15
<212> TYPE: PRT
<213> ORGANISM: Artificial sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic peptide

<400> SEQUENCE: 6

Ser Thr Pro Glu Lys Glu Gly Glu Leu Glu Gly Thr Thr Thr Lys
1               5                   10                  15


<210> SEQ ID NO 7
<211> LENGTH: 18
<212> TYPE: PRT
<213> ORGANISM: Artificial sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic peptide

<400> SEQUENCE: 7

Ser Gln Leu Glu Thr Pro Glu Thr Leu Leu Gly Ser Thr Glu Glu Lys
1               5                   10                  15

Pro Leu


<210> SEQ ID NO 8
<211> LENGTH: 4
<212> TYPE: PRT
```

<213> ORGANISM: Artificial sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic peptide

<400> SEQUENCE: 8

Val Phe Cys Thr
1

<210> SEQ ID NO 9
<211> LENGTH: 16
<212> TYPE: PRT
<213> ORGANISM: Artificial sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic peptide

<400> SEQUENCE: 9

Asn Gln Pro Gln Ala Pro Gly Val Glu Ala Ser Gly Ala Gly Glu Ala
1 5 10 15

<210> SEQ ID NO 10
<211> LENGTH: 18
<212> TYPE: PRT
<213> ORGANISM: Artificial sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic peptide
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (8)..(8)
<223> OTHER INFORMATION: Xaa = unknown amino acid

<400> SEQUENCE: 10

Leu Pro Ala Gln Val Ala Phe Xaa Pro Tyr Ala Pro Glu Pro Gly Ser
1 5 10 15

Thr Cys

<210> SEQ ID NO 11
<211> LENGTH: 13
<212> TYPE: PRT
<213> ORGANISM: Artificial sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic peptide
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (2)..(2)
<223> OTHER INFORMATION: Xaa = unknown amino acid

<400> SEQUENCE: 11

Ile Xaa Pro Gly Phe Gly Val Ala Tyr Pro Ala Leu Glu
1 5 10

<210> SEQ ID NO 12
<211> LENGTH: 4
<212> TYPE: PRT
<213> ORGANISM: Artificial sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic peptide

<400> SEQUENCE: 12

Leu Cys Ala Pro
1

<210> SEQ ID NO 13
<211> LENGTH: 7
<212> TYPE: PRT
<213> ORGANISM: Artificial sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic peptide

<400> SEQUENCE: 13

Val Pro His Leu Pro Ala Asp
1 5

<210> SEQ ID NO 14
<211> LENGTH: 15
<212> TYPE: PRT
<213> ORGANISM: Artificial sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic peptide
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (9)..(10)
<223> OTHER INFORMATION: Xaa = unknown amino acid
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (13)..(13)
<223> OTHER INFORMATION: Xaa = unknown amino acid

<400> SEQUENCE: 14

Gly Ser Gln Gly Pro Glu Gln Gln Xaa Xaa Leu Ile Xaa Ala Pro
1 5 10 15

<210> SEQ ID NO 15
<211> LENGTH: 9
<212> TYPE: PRT
<213> ORGANISM: Artificial sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic peptide

<400> SEQUENCE: 15

Leu Val Pro His Leu Gly Asp Arg Glu
1 5

<210> SEQ ID NO 16
<211> LENGTH: 27
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic primer

<400> SEQUENCE: 16

agggagaaga gagatagtgt gtgtccc 27

<210> SEQ ID NO 17
<211> LENGTH: 41
<212> TYPE: DNA
<213> ORGANISM: Artificial sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic primer

<400> SEQUENCE: 17

aagcttggcc aggatccagc tgactgactg atcgcgagat c 41

<210> SEQ ID NO 18
<211> LENGTH: 41
<212> TYPE: DNA
<213> ORGANISM: Artificial sequence
<220> FEATURE:
<223> OTHER INFORMATION: Antisense primer

<400> SEQUENCE: 18

gatctcgcga tcagtcagtc agctggatcc tggccaagct t 41

<210> SEQ ID NO 19
<211> LENGTH: 38
<212> TYPE: DNA
<213> ORGANISM: Artificial sequence
<220> FEATURE:

-continued

<223> OTHER INFORMATION: Synthetic primer

<400> SEQUENCE: 19

cacagggatc catagctgtc tggcatgggc ctctccac 38

<210> SEQ ID NO 20
<211> LENGTH: 44
<212> TYPE: DNA
<213> ORGANISM: Artificial sequence
<220> FEATURE:
<223> OTHER INFORMATION: Antisense primer

<400> SEQUENCE: 20

cccggtacca gatctctatt atgtggtgcc tgagtcctca gtgc 44

<210> SEQ ID NO 21
<211> LENGTH: 19
<212> TYPE: DNA
<213> ORGANISM: Artificial sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic primer

<400> SEQUENCE: 21

gatccagaat tcataatag 19

<210> SEQ ID NO 22
<211> LENGTH: 19
<212> TYPE: DNA
<213> ORGANISM: Artificial sequence
<220> FEATURE:
<223> OTHER INFORMATION: Antisense primer

<400> SEQUENCE: 22

gtacctatta tgaattctg 19

<210> SEQ ID NO 23
<211> LENGTH: 31
<212> TYPE: DNA
<213> ORGANISM: Artificial sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic primer

<400> SEQUENCE: 23

gcaccacata atagagatct ggtaccggga a 31

<210> SEQ ID NO 24
<211> LENGTH: 25
<212> TYPE: DNA
<213> ORGANISM: Artificial sequence
<220> FEATURE:
<223> OTHER INFORMATION: Antisense primer

<400> SEQUENCE: 24

cccggtacca gatctctatt atgtg 25

<210> SEQ ID NO 25
<211> LENGTH: 29
<212> TYPE: DNA
<213> ORGANISM: Artificial sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic primer

<400> SEQUENCE: 25

tacgagctcg gccatagctg tctggcatg 29

<210> SEQ ID NO 26

-continued

<211> LENGTH: 29
<212> TYPE: DNA
<213> ORGANISM: Artificial sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic primer

<400> SEQUENCE: 26

atagagctct gtggtgcctg agtcctcag 29

The invention claimed is:

**1**. A protein comprising

(a) a human tumor necrosis factor (TNF)-binding soluble fragment of an insoluble human TNF receptor, wherein the insoluble human TNF receptor (i) specifically binds human TNF, (ii) has an apparent molecular weight of about 75 kilodaltons on a non-reducing SDS-polyacrylamide gel, and (iii) comprises the amino acid sequence LPAQVAFXPYAPEPGSTC (SEQ ID NO: 10); and

(b) all of the domains of the constant region of a human immunoglobulin IgG heavy chain other than the first domain of said constant region;

wherein said protein specifically binds human TNF.

**2**. The protein of claim **1**, wherein the soluble fragment comprises the peptides LCAP (SEQ ID NO: 12) and VFCT (SEQ ID NO: 8).

**3**. The protein of claim **2**, wherein the soluble fragment further comprises the peptide LPAQVAFXPYAPEPGSTC (SEQ ID NO: 10).

**4**. The protein of claim **1**, wherein said human immunoglobulin IgG heavy chain is $IgG_1$.

**5**. The protein of claim **4**, wherein said domains of the constant region of the human immunoglobulin heavy chain consist essentially of the immunoglobulin amino acid sequence encoded by pCD4-Hγ1 vector (deposited at Deutschen Sammlung von Mikroorganismen und Zellkulturen GmbH (DSM) in Braunschweig, FRG under No. DSM 5314).

**6**. A pharmaceutical composition comprising the protein of claim **4** and a pharmaceutically acceptable carrier material.

**7**. The protein of claim **1**, wherein the protein is purified.

**8**. The protein of claim **1**, wherein the protein is produced by CHO cells.

**9**. The protein of claim **1**, wherein the protein consists of (a) the soluble fragment of the receptor and (b) all of the domains of the constant region of the human immunoglobulin IgG heavy chain other than the first domain of the constant region.

**10**. The protein of claim **1**, wherein said domains of the constant region of the human immunoglobulin heavy chain consist essentially of the immunoglobulin amino acid sequence encoded by pCD4-Hγ1 vector (deposited at Deutschen Sammlung von Mikroorganismen and Zellkulturen GmbH (DSM) in Braunschweig, FRG under No. DSM 5314) or by pCD4-Hγ3 vector (deposited at Deutschen Sammlung von Mikroorganismen und Zellkulturen GmbH (DSM) in Braunschweig, FRG under No. DSM 5523).

**11**. The protein of claim **1**, wherein the protein consists essentially of the extracellular region of the insoluble human TNF receptor and all the domains of the constant region of a human $IgG_1$ immunoglobulin heavy chain other than the first domain of the constant region.

**12**. A pharmaceutical composition comprising the protein of claim **11** and a pharmaceutically acceptable carrier material.

**13**. A protein comprising

(a) a human tumor necrosis factor (TNF)-binding soluble fragment of an insoluble human TNF receptor, wherein the insoluble human TNF receptor (i) specifically binds human TNF, (ii) has an apparent molecular weight of about 75 kilodaltons on a non-reducing SDS-polyacrylamide gel, and (iii) comprises the amino acid sequences LPAQVAFXPYAPEPGSTC (SEQ ID NO: 10), LCAP (SEQ ID NO: 12), VFCT (SEQ ID NO: 8), NQPQAPGVEASGAGEA (SEQ ID NO: 9) and VPHLPAD (SEQ ID NO: 13),

wherein the soluble fragment comprises the peptides LCAP (SEQ ID NO: 12) and VFCT (SEQ ID NO: 8); and

(b) all of the domains of the constant region of a human $IgG_1$ heavy chain other than the first domain of the constant region;

wherein said protein specifically binds human TNF.

**14**. The protein of claim **13**, wherein the protein is purified.

**15**. The protein of claim **13**, wherein the protein is produced by CHO cells.

**16**. The protein of claim **13**, wherein the protein consists of (a) the soluble fragment of the receptor and (b) all of the domains of the constant region of the human $IgG_1$ heavy chain other than the first domain of the constant region.

**17**. The protein of claim **13**, wherein the soluble fragment further comprises the peptide LPAQVAFXPYAPEPGSTC (SEQ ID NO: 10).

**18**. A protein encoded by a polynucleotide which comprises two nucleic acid subsequences,

(a) one of said subsequences encoding a human TNF-binding soluble fragment of an insoluble human TNF receptor protein having an apparent molecular weight of about 75 kilodaltons on a non-reducing SDS-polyacrylamide gel, said soluble fragment comprising the amino acid sequence LPAQVAFXPYAPEPGSTC (SEQ ID NO: 10), and

(b) the other of said subsequences encoding all of the domains of the constant region of the heavy chain of a human IgG immunoglobulin other than the first domain of said constant region,

wherein said protein specifically binds human TNF.

**19**. The protein of claim **18**, wherein the soluble fragment comprises the peptides LCAP (SEQ ID NO: 12) and VFCT (SEQ ID NO: 8).

**20**. The protein of any one of claim **18** or **19**, wherein said human immunoglobulin heavy chain is $IgG_1$.

**21**. The protein of claim **20**, wherein said domains of the constant region of the human immunoglobulin heavy chain consist essentially of the immunoglobulin amino acid sequence encoded by the DNA insert of pCD4-Hγ1 vector (deposited at Deutschen Sammlung von Mikroorganismen und Zellkulturen GmbH (DSM) in Braunschweig, FRG under No. DSM 5314).

22. The protein of claim 18, wherein the protein is purified.

23. The protein of claim 18, wherein the protein is produced by CHO cells.

24. The protein of claim 18, wherein the protein consists of (a) the soluble fragment of the receptor and (b) all of the domains of the constant region of the human $IgG_1$ heavy chain other than the first domain of the constant region.

25. The protein of claim 18, wherein said domains of the constant region of the human immunoglobulin heavy chain consist essentially of the immunoglobulin amino acid sequence encoded by pCD4-Hγ1 vector (deposited at Deutschen Sammlung von Mikroorganismen und Zellkulturen GmbH (DSM) in Braunschweig, FRG under No. DSM 5314) or by pCD4-Hγ3 vector (deposited at Deutschen Sammlung von Mikroorganismen und Zellkulturen GmbH (DSM) in Braunschweig, FRG under No. DSM 5523).

26. A protein consisting of

(a) a human tumor necrosis factor (TNF)-binding soluble fragment of an insoluble human TNF receptor, wherein the insoluble human TNF receptor (i) specifically binds human TNF, and (ii) has an apparent molecular weight of about 75 kilodaltons on a non-reducing SDS-polyacrylamide gel and (iii) comprises the amino acid sequence LPAQVAFXPYAPEPGSTC (SEQ ID NO: 10),

wherein the soluble fragment comprises the peptides LCAP (SEQ ID NO:12) and VFCT (SEQ ID NO:8), and

(b) all of the domains of the constant region of a human $IgG_1$ heavy chain other than the first domain of the constant region,

wherein the protein specifically binds human TNF, and

wherein the protein is produced by CHO cells.

27. The protein of claim 26, wherein the soluble fragment comprises the peptide LPAQVAFXPYAPEPGSTC (SEQ ID NO: 10).

28. The protein of claim 26, wherein the protein is purified.

29. A pharmaceutical composition comprising the protein of any of claim 1, 18, 26, or 27 and a pharmaceutically acceptable carrier material.

30. A protein comprising

(a) human tumor necrosis factor (TNF) binding soluble fragment of the amino acid sequence encoded by the cDNA insert of the plasmid deposited with the ATCC on Oct. 17, 2006 under accession number PTA 7942,

(b) all of the domains of the constant region of a human immunoglobulin IgG heavy chain other than the first domain of said constant region;

wherein said protein specifically binds human TNF.

31. The protein of claim 30, consisting of the soluble fragment and all the domains of the constant region of the human immunoglobulin IgG heavy chain other than the first domain of said constant region.

32. The protein of claim 30, wherein the protein is expressed by a mammalian host cell.

33. The protein of claim 32, wherein the mammalian host cell is a CHO cell.

34. The protein of claim 32, consisting of the soluble fragment and all the domains of the constant region of the human immunoglobulin IgG heavy chain other than the first domain of said constant region.

35. The protein of claim 30, wherein the protein consists essentially of the extracellular region of the human tumor necrosis factor (TNF) receptor amino acid sequence encoded by the cDNA insert, and all the domains of the constant region of a human $IgG_1$ immunoglobulin heavy chain other than the first domain of the constant region.

36. A pharmaceutical composition comprising the protein of claim 35 and a pharmaceutically acceptable carrier material.

* * * * *

# EXHIBIT 2

US008163522B1

(12) **United States Patent**
**Brockhaus et al.**

(10) **Patent No.: US 8,163,522 B1**
(45) **Date of Patent: Apr. 24, 2012**

(54) **HUMAN TNF RECEPTOR**

(75) Inventors: **Manfred Brockhaus**, Bettingen (CH); **Reiner Gentz**, Rheinfelden (DE); **Dembic Zlatko**, Basel (CH); **Werner Lesslauer**, Basel (CH); **Hansruedi Lotscher**, Mohlin (CH); **Ernst-Jurgen Schlaeger**, Efringen-Kirchen (DE)

(73) Assignee: **Hoffman-LaRoche Inc.**, Nutley, NJ (US)

( * ) Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 0 days.

(21) Appl. No.: **08/444,791**

(22) Filed: **May 19, 1995**

**Related U.S. Application Data**

(60) Division of application No. 08/095,640, filed on Jul. 21, 1993, now Pat. No. 5,610,279, which is a continuation of application No. 07/580,013, filed on Sep. 10, 1990, now abandoned.

(30) **Foreign Application Priority Data**

| | | |
|---|---|---|
| Sep. 12, 1989 | (CH) | 3319/89 |
| Mar. 8, 1990 | (CH) | 746/90 |
| Apr. 20, 1990 | (CH) | 1347/90 |
| Aug. 31, 1990 | (EP) | 90116707 |

(51) **Int. Cl.**

| | |
|---|---|
| ***C12N 15/12*** | (2006.01) |
| ***C12N 15/63*** | (2006.01) |
| ***C12N 5/10*** | (2006.01) |
| ***C12P 21/00*** | (2006.01) |

(52) **U.S. Cl.** ..... **435/69.9**; 536/23.4; 435/320.1; 435/325; 435/358

(58) **Field of Classification Search** ..... 530/350, 530/385, 388.22, 387.1, 866, 867, 391.1; 636/23.53, 23.1; 435/69.7, 70.21, 320.1; 536/23.4

See application file for complete search history.

(56) **References Cited**

U.S. PATENT DOCUMENTS

| | | | | |
|---|---|---|---|---|
| 4,593,002 | A | | 6/1986 | Dulbecco |
| 4,675,285 | A | | 6/1987 | Clark et al. |
| 4,729,326 | A | | 3/1988 | Richter |
| 4,769,326 | A | | 9/1988 | Rutler |
| 4,770,995 | A | | 9/1988 | Rubin et al. |
| 4,816,567 | A | | 3/1989 | Cabilly et al. |
| 4,894,439 | A | | 1/1990 | Dorin et al. |
| 4,912,044 | A | | 3/1990 | Jacob et al. |
| 4,935,233 | A | | 6/1990 | Bell et al. |
| 4,948,875 | A | | 8/1990 | Tanaka et al. |
| 4,963,354 | A | | 10/1990 | Shepard et al. |
| 4,965,271 | A | | 10/1990 | Mandell et al. |
| 5,055,447 | A | | 10/1991 | Palladino et al. |
| 5,073,627 | A | | 12/1991 | Curtis et al. |
| 5,075,222 | A | | 12/1991 | Hannum et al. |
| 5,098,702 | A | | 3/1992 | Zimmerman et al. |
| 5,098,833 | A | | 3/1992 | Lasky et al. |
| 5,116,964 | A | | 5/1992 | Capon et al. |
| 5,118,500 | A | | 6/1992 | Hanel et al. |
| 5,136,021 | A | | 8/1992 | Dembinski et al. |
| 5,155,027 | A | | 10/1992 | Sledziewski et al. |
| 5,211,945 | A | | 5/1993 | Wallach et al. |
| 5,223,395 | A | | 6/1993 | Gero |
| 5,225,538 | A | | 7/1993 | Capon et al. |
| 5,258,498 | A | | 11/1993 | Huston et al. |
| 5,264,416 | A | | 11/1993 | Park et al. |
| 5,270,038 | A | | 12/1993 | Klimpel et al. |
| 5,336,603 | A | | 8/1994 | Capon et al. |
| 5,344,915 | A | * | 9/1994 | LeMaire et al. ..... 530/350 |
| 5,350,683 | A | | 9/1994 | Sims et al. |
| 5,359,032 | A | | 10/1994 | Dayer et al. |
| 5,395,760 | A | * | 3/1995 | Smith et al. ..... 435/240.1 |
| 5,428,130 | A | * | 6/1995 | Capon et al. ..... 530/350 |
| 5,447,851 | A | | 9/1995 | Beutler et al. |
| 5,455,165 | A | | 10/1995 | Capon et al. |
| 5,478,925 | A | | 12/1995 | Wallach et al. |
| 5,512,544 | A | | 4/1996 | Wallach et al. |
| 5,514,582 | A | | 5/1996 | Capon et al. |
| 5,599,905 | A | | 2/1997 | Mosley et al. |
| 5,605,690 | A | | 2/1997 | Jacobs et al. |
| 5,610,279 | A | | 3/1997 | Brockhaus et al. |
| 5,633,145 | A | | 5/1997 | Feldmann et al. |
| 5,639,597 | A | | 6/1997 | Lauffer et al. |

(Continued)

FOREIGN PATENT DOCUMENTS

| | | |
|---|---|---|
| AU | 58976 | 1/1991 |

(Continued)

OTHER PUBLICATIONS

Lee et al., Science, vol. 239: 1284-1291, Generation of cDNA Probes Directed by Amino Acid Sequence: Cloning of Urate Oxidase, Mar. 11, 1988.*

Wozney, Methods in Enzymology, vol. 182:738-751, "Using Purified Protein to Clone Its Gene", 1990.*

Monnat, Jr "Molecular Analysis of Spontaneous Hypoxanthine Phosphoribosyltransferase Mutations in Thioguanine-resistant HL-60 Human Leukemia Cells" Cancer Research, 49:81-87, 1989.*

Peppel, K., et al., J. Exp. Med., 174, pp. 1483-1489 (1991).

Zettlmeissl, G., et al., DNA & Cell Biology, 9, pp. 347-353 (1990).

Loetscher et al, *J. of Bio. Chem.*, 266(27), pp. 18324-18329 (1991).

Nature Biotechnology, 15 (1997) p. 13.

Stauber et al., "Human tumor necrosis factor-α receptor-purification by immunoaffinity chromatography and initial characterization", J. Bio. Chem. 263:19098-19104 (1988).

(Continued)

*Primary Examiner* — Ronald Schwadron

(74) *Attorney, Agent, or Firm* — Marshall, Gerstein & Borun LLP

(57) **ABSTRACT**

The present invention is concerned with non-soluble proteins and soluble or insoluble fragments thereof, which bind TNF, in homogeneous form, as well as their physiologically compatible salts, especially those proteins having a molecular weight of about 55 or 75 kD (non-reducing SDS-PAGE conditions), a process for the isolation of such proteins, antibodies against such proteins, DNA sequences which code for non-soluble proteins and soluble or non-soluble fragments thereof, which bind TNF, as well as those which code for proteins comprising partly of a soluble fragment, which binds TNF, and partly of all domains except the first of the constant region of the heavy chain of human immunoglobulins and the recombinant proteins coded thereby as well as a process for their manufacture using transformed pro- and eukaryotic host cells.

**10 Claims, 16 Drawing Sheets**

U.S. PATENT DOCUMENTS

| | | | |
|---|---|---|---|
| 5,695,953 | A | 12/1997 | Wallach et al. |
| 5,705,364 | A | 1/1998 | Etcheverry et al. |
| 5,712,155 | A | 1/1998 | Smith et al. |
| 5,721,121 | A | 2/1998 | Etcheverry et al. |
| 5,808,029 | A | 9/1998 | Brockhaus et al. |
| 5,811,261 | A | 9/1998 | Wallach et al. |
| 5,863,786 | A | 1/1999 | Feldmann et al. |
| 5,945,397 | A | 8/1999 | Smith et al. |
| 5,981,701 | A | 11/1999 | Wallach et al. |
| RE36,755 | E | 6/2000 | Smith et al. |
| 6,143,866 | A | 11/2000 | Brewer et al. |
| 6,165,476 | A | 12/2000 | Strom et al. |
| 6,201,105 | B1 | 3/2001 | Smith et al. |
| 6,221,675 | B1 | 4/2001 | Hauptmann et al. |
| 6,271,346 | B1 | 8/2001 | Hauptmann et al. |
| 6,294,352 | B1 | 9/2001 | Hauptmann et al. |
| 6,541,610 | B1 | 4/2003 | Smith |
| 6,541,620 | B1 | 4/2003 | Brewer et al. |
| 6,572,852 | B2 | 6/2003 | Smith et al. |
| 6,858,409 | B1 | 2/2005 | Thompson et al. |
| 7,253,264 | B1 | 8/2007 | Lauffer et al. |
| 2003/0064480 | A1 | 4/2003 | Lauffler et al. |

FOREIGN PATENT DOCUMENTS

| | | | |
|---|---|---|---|
| EP | 120694 | | 10/1984 |
| EP | 227110 | | 7/1987 |
| EP | 230574 | | 8/1987 |
| EP | 269455 | | 6/1988 |
| EP | 315 062 | | 10/1988 |
| EP | 308 378 | | 3/1989 |
| EP | 314317 | | 5/1989 |
| EP | 325 224 | | 7/1989 |
| EP | 325262 | | 7/1989 |
| EP | 0334165 | | 9/1989 |
| EP | 393 438 | | 4/1990 |
| EP | 0 394 827 | A1 | 10/1990 |
| EP | 398 327 | | 11/1990 |
| EP | 412 486 | | 2/1991 |
| EP | 414178 | | 2/1991 |
| EP | 418 014 | | 3/1991 |
| EP | 422 339 | | 4/1991 |
| EP | 433 900 | | 6/1991 |
| EP | 460846 | | 12/1991 |
| EP | 526452 | | 2/1993 |
| EP | 526905 | | 2/1993 |
| EP | 0 567 566 | B1 | 11/1993 |
| EP | 568925 | | 11/1993 |
| EP | 606869 | | 7/1994 |
| GB | 2218101 | A | 10/1989 |
| GB | 2218101 | | 11/1989 |
| GB | 2 246 569 | | 2/1992 |
| JP | 61-293924 | | 12/1986 |
| JP | 02-154695 | | 6/1990 |
| WO | 89/02922 | * | 4/1989 |
| WO | WO 89 09622 | | 10/1989 |
| WO | WO 91/02078 | | 2/1991 |
| WO | 91/03553 | | 3/1991 |
| WO | WO 91/17184 | | 11/1991 |
| WO | WO 91/08298 | | 12/1991 |
| WO | WO 92/08495 | | 5/1992 |
| WO | WO 92/13095 | | 8/1992 |
| WO | WO 93/07863 | | 4/1993 |
| WO | WO 93/19777 | | 10/1993 |
| WO | WO 94/06476 | | 3/1994 |

OTHER PUBLICATIONS

S ckinger et al., "Purification and bi logic charact rization of a specific tumor necrosis factor α inhibitor", J. Bio. Chem. 264:11966-11973 (1989).

Engelmann et al., "A tumor necrosis factor-binding protein purified to homogeneity from human urine protects cells from tumor necrosis factor toxicity", J. Bio. Chem. 264:11974-11980 (1989).

Hohmann et al., "Two different cell types have different major rec ptors for human tumor necrosis factor (TNFα)", J. Bio. Ch m. 264: 14927-14934 (1989).

Smith et al., "A rec ptor for tumor necrosis factor defines an unusual family of cellular and viral prot ins", Science 248:1019-1023 (1990).

Heller et al., "Complementary DNA cloning of a receptor for tumor necrosis factor and demonstraction of a shed form of the receptor", Proc. Natl. Acad. Sci. U.S.A. 87:6151-6155 (1990).

Novick et al., "Soluble cytokine receptors are present in normal human urine", J. Exp. Med. 170:1409-1414 (1989).

Engelmann et al., "Two tumor necrosis factor-binding proteins purified from human urine", J. Bio. Chem. 265:1531-1536 (1990).

Schall et al., "Molecular cloning and expression of a receptor for human tumor necrosis factor", Cell 61:361-370 (1990).

Seckinger et al., "Tumor necrosis factor inhibitor: purification, $NH_2$-terminal amino acid sequence and evidence for anti-inflammatory and immunomodulatory activities", Eur. J. Immunol. 20:1167-1174 (1990).

Hohmann et al., "Expression of the types A and B tumor necrosis factor (TNT) receptors is independently regulated, and both receptors mediate activiation of the transcription factor NF-kB", Bio. Ch m. 265:22409-22417 (1990).

Espevik et al., "Characterization of binding and biological effects of monoclonal antibodies against a human tumor necrosis factor receptor", J. Exp. Med. 171:415-426 (1990).

Porteu and Nathan, "Shedding of tumor necrosis factor receptors by activated human neutrophils", J. Exp. Med. 172:599-607 (1990).

Eng lmann et al., "Antibodies to a soluble form of a tumor necrosis factor (TNF) receptor have TNF-like activity", J. Bio. Chem. 265:14497-14504 (1990).

Seckinger et al., "Characterization of a tumor necrosis factor α (TNF-α) inhibitor: evidence of immunological cross-activity with the TNF receptor", Proc. Natl. Sci. USA 87:5188-5192 (1990).

Gray et al., "Cloning of human tumor necrosis factor (TNF) receptor cDNA and expression of recombinant soluble TNF-binding protein", Proc. Natl. Sci. USA 87:76380-7384 (1990).

Loetscher et al., "Molecular cloning and expression of the human 55 kd tumor necrosis factor receptor", Cell 61:351-359 (1990).

Peppel et al., "Chimaeric TNF-receptor-IgC molecule acts as soluble inhibitor of TNF mediated cytotoxicity", Journal of Cellular Biochem., Abstract, 20th Annual Meetings, Keystone Symposia on Molecular and Cellular Biology, p. 118, Suppl m nt 15F (1991).

Olsson et al., "Isolation and characterization of a tumor necrosis factor binding protein from urine", Eur. J. Ha matol. 42:270-275 (1989).

Capon et al., "Designing CD4 immunoadhesions for AIDS therapy", Nature 337:525-530 (Feb. 9, 1989).

Abstract 92-009794/02 (1992) for EP 464 533.

Official Communication relating to an Opposition in EP Application No. 99 100 703.0.

U.S. Appl. No. 08/478,995, Lauffler, Leander et al.

Abraham et al., p55 Tumor Necrosis Factor Receptor Fusion Protein in the Treatment of Patients With Severe Sepsis and Septic Shock: ΔΔΔΔΔ JAMA, 19:1531-1538 (1997).

Abraham et al., Lenercept (p55 TuMor Necrosis Factor Receptor Fusion Protein) in Severe Sepsis and Early Septic Shock: A Randomized, Double-Blind, Placebo-Controlled, Multicenter Phase III Trial With 1,342 Patients, Crit Care Med,. 29:503-510 (2001).

Aggarwal et al., Characterization of Receptors for Human Tumour Necrosis Factor and Their Regulation by γ-Interferon, Nature, 318:665-667 (1985).

Aggarwal et al., Induction of Receptors for Tuthor Necrosis Factor-α by Interferons Is Not a Major Mechanism for Their Synergistic Cytotoxic Response, J. Biol. Chem., 262:10000-10007 (1987).

Aggarwal et al., Human tumour necrosis factors: structure and receptor interactions, in Tumor necrosis factor and related cytotoxins, pp. 39-51, (Ciba Foundation symposium 131), Wiley, Chichester (1987).

Arenzana-Seisdedos et al., Immunoregulatory Mediators in the Pathogenesis of Rheumotoid Arthritis, Scand. J. Rheumatol., Supplement 66:13-17 (1987).

Aruffo et al., Molecular Cloning of a CD28 cDNA by a High-Efficiency COS Cell Expression. System, Proc. Natl. Acad. Sci. USA, 84:8573-8577 (1987).

Ashkenazi et al., Protection Against Endotoxic Shock by a Tumor Necrosis Factor Receptor Immunoadhesin, Proc. Natl. Acad. Sci., U.S.A. 88:10535-10539 (1991).

Ayala, Modem Genetics, Benjamin/Cummings, Publ. Co., Menlo Park CA, p. 45, (1980).

Baglioni et al., Binding of Human Tumor Necrosis Factor to High Affinity Receptors on HeLa and Lymphoblastoid Cells Sensitive to Growth Inhibition, J. Biol. Chem., 260:13395-13397 (1985).
Benjamini et al., Antibody Structure, in Immunology: A Short Course, 3rd ed., Wiley-Liss New York, 61-69 (1996).
Branellec et al., TNF: Antitumoral Agent at the Border Lines of Immunity and Inflammation, Path. Biol., 39:230-239 (1991).
Brockhaus et al., Identification of Two Types of Tumor Necrosis Factor Receptors on Human Cell Lines by Monoclonal Antibodies, Proc. Natl. Acad. Sci. USA, 87:3127-3131 (1990).
Carter et al., Purification, Cloning, Expression and Biological Characterization of an Interleukin-1 Receptor Antagonist Protein, Nature, 344:633-638 (1990).
Carpenter et al., Epidermal Growth Factor, J. Biol. Chem., 265:7709-7712 (1990).
Carpenter, Receptors for Epidermal Growth Factor and Other Polypeptide Mitogens, Ann. Rev. Biochem., 56:881-914 (1987).
Casadei et al., Expression and Secretion of Aequorin as a Chimeric Antibody by Means of a Mammalian Expression Vector, Proc. Natl. Acad. Sci., U.S.A. 87:2047-2051 (1990).
Coffman et al., The Role of Helper T Cell Products in Mouse B Cell Differentiation and Isotype Regulation, Immunol. Rev., 102:5-28 (1988).
Creasey et al., A High Molecular Weight Component of the Human Tumor Necrosis Factor Receptor is Associated With Cytotoxicity, Proc. Natl. Acad. Sci. USA, 84:3293-3297 (1987).
Dayer, Chronic Inflammatory Joint Diseases: Natural Inhibitors of Interleukin 1 and Tumor Necrosis Factor α, J. Rheumatol, 18 (Suppl. 27): 71-75 (1991).
Dower et al., Human Cytokine Receptors, J. Clin. Immunol., 10:289-299 (1990).
Eisenberg et al., Primary Structure and Functional Expression From Complementary DNA of a Human Interleukin-1 Receptor Antagonist, Nature, 343:341-346 (1990).
Ellison et al., The Nucleotide Sequence of a Human Immunoglobulin Cy1 Gene, Nucleic Acids Res. 10(13): 4071-79 (1982).
Esmon, The Roles of Protein C and Thrombomodulin in the Regulation of Blood Coagulation, J. Biol. Chem., 264:4743-4746 (1989).
European Search Report for EP 97 12 0664, dated Mar. 9, 1998.
Fell et al., Genetic Construction and Characterization of a Fusion Protein Consisting of a Chimeric F(ab') With Specificity for Carcinomas and Human IL-2, J. Immunol., 146:2446-2452 (1991).
Fernandez-Botran et al., A Soluble, High-Affinity, Interleukin-4-Binding Protein is Present in the Biological Fluids of Mice, Proc. Natl. Acad. Sci., 87:4202-4206 (1990).
Fernandez-Botran, Soluble Cytokine Receptors: Their Role in Immunoregulation, The FASEB Journal, 5:2567-2574 (1991).
Ferrante et al., Inhibition of Tumour Necrosis Factor Alpha (TNF-α)-Induced Neutrophil Respiratory Burst by a TNF Inhibitor, Immunology, 72:440-442 (1991).
Fisher et al., Cloning and Expression of Human Tissue Factor cDNA, Thrombosis Research, 48:89-99 (1987).
Fisher et al., Treatment of Septic Shock with the Tumor Necrosis Factor Receptor: Fc Fusion Protein, New Eng. J. Med., 334:1697-1702 (1996).
Foley et al., An Inhibitor of the Toxicity of Tumour Nectrosis Factor in the Serum of Patients with Sarcoidosis, Tuberculosis and Crohn's Disease, Clin. Exp. Immunol, 80:395-399 (1990).
Fomsgaard et al., Preliminary Study on Treatment of Septic Shock Patients With Antilipopolysaccharide IgG from Blood Donors, Scand. J. Infect. Dis., 21:697-708 (1989).
Garcia et al., High Sensitivity of Transgenic Mice Expressing Soluble TNFR1 Fusion Protein to Mycobacterial Infections: Synergistic Action of TNF and IFN-γ in the Differentiation of Protective Granulomas, Eur. J. Immunol., 27:3182-3190 (1997).
Gascoigne et al., Secretion of a Chimeric T-Cell Receptor-Immunoglobulin Protein, Proc. Natl. Acad. Sci USA, 84:2936-2940 (1987).
Gehr et al., Both Tumor Necrosis Factor Receptor Types Mediate Proliferative Signals in Human MononuClear Cell Activation, J. Immunol., 149:911-917 (1992).
Gillies et al., Targeting Human Cytotoxic T Lymphocytes to Kill Heterologous Epidermal Growth Factor Receptor-Bearing Tumor Cells, J. Immunol., 144:1067-1071 (1991).
Goodman, Identification of Antigenic Determinants, in Basic & Clinical Immunol., 24-25 (1982).
Goodman, Immunogenicity & Antigenic Specificity, in Basic & Clinical Immunol., 101-108 (1991).
Goodwin et al., Molecular cloning and Expression of the Type 1 and Type 2 Murine Receptors for Tumor Necrosis Factor, Molecular and Cellular Biology, 11:3020-3026 (1991).
Gray et al, Cloning and Expression of cDNA for Human Lymphotoxin, a Lymphokine With Tumour Necrosis Activity, Nature, 312:721-724 (1984).
Gray et al., Cloning of human tumor necrosis factor (TNF) receptor cDNA and expression of recombinant soluble TNF-binding protein, Proc. Natl. ACad. Sci. 87: 7380-84 (1990).
Grundmann et al., Characterization of cDNA Coding for Human Factor Xllla, Proc. Natl. Acad. Sci. USA, 83:8024-8028 (1986).
Haak-Frendscho et al., Inhibition of TNF by a TNF Receptor Immunoadhesin, J. Immunol., 152:1347-1353 (1994).
Hannum et al., Interleukin-1 Receptor Antagonist Activity of a Human Interleukin-1 Inhibitor, Nature, 343:336-340 (1990).
Heflin et al., Prevention by Granulocyte Depletion of Increased Vascular Permeability of Sheep Lung Following Endotoxemia, J. Clin. Invest., 68:1253-1260 (1981).
Heller et al., Amplified Expression of Tumor Necrosis Factor Receptor in Cells Transfected With Epstein-Barr Virus Shuttle Vector cDNA Libraries, J. Biol. Chem., 265:5708-5717 (1990).
Heller et al., Amplified Expression of. The Turrior Necrosis Factor Receptor in Lymphoblastoid Cells Transfected with HeLa Cell-CDNA Expression Abstract WA 142, Napa Valley Conference 1989.
Himmler et al., Molecular Cloning and Expression of Human and Rat Tumor Necrosis Factor Receptor Chain (p60) and Its Soluble Derivative, Tumor Necrosis Factor-Binding Protein, DNA and Cell Biology, 9:705-715 (1990).
Hobart, The Immune System: A Course on the Molecular and Cellular Basis of immunity, Blackwell Scientific Pubs, p. 7 (1975).
Holtmann et al., Down Regulation of the Receptors for Tumor Necrosis Factor by Intereukin 1 and 4β-Phorbol-12-Myristate-13-Acetate, J. Immunol., 139:1161-1167 (1987).
Hsu et al., Differential Expression and Ligand Binding Properties of Tumor Necrosis Factor Receptor Chimeric Mutants, J. Biol. Chem., 268:16430-16436(1992).
Idzerda et al., Human Interleukin 4 Receptor Confers Biological Responsiveness and Defines a Novel Receptor Superfamily, J. Exp. Med., 171:861-873 (1990).
Imamura et al., Expression of Tumor Necrosis Factor Receptors on Human Monocytes and Internalization of Receptor Bound Ligand, J. Immunol., 139:2989-2992 (1987).
Ishikura et al., Differential Biologic Effects Resulting From Bimodal Binding of Recombinant Human Tumor Necrosis Factor to Myeloid Leukemia Cells, Blood, 73:419-424 (1989).
Israel et al., Binding of Human TNF-α to High-Affinity Cell Surface Receptors: Effect of IFN, Immunology Letters, 12:217-224(1986).
Jacobs et al., Pharmacokinetic Parameters and Biodistribution of Soluble Cytokine Receptors, International Review of Expermental Pathology, 34B:123-135 (1993).
Jones et al, Structure of Tumour Necrosis Factor, Nature, 338:225-228 (1989).
Kaczmarski et al., The Cytokine Receptor Superfamily, Blood Reviews, 5:193-203 (1991).
Kaushansky, Structure-Function Relationships of the Hematopoietic Growth Factors, Proteins: Structure, Function & Genetics, 12:1-9 (1992).
Keegan et al., The Interleukin-4 Receptor: Signal Transduction by a Hematopoietin Receptor, Journal of Leukocyte Biology, 55:272-279 (1994).
Keegan et al., Interleukin 4 Receptor: Signaling Mechanisms, Immunology Today, 15:423-432 (1994).
Kleinau et al., Importance of CD23 for Collagen-Induced Arthritis: Delayed Onset and Reduced Severity in CD23-Deficient Mice, J. Immunol., 162:4266-4270 (1999).

Klinkert et al., TNF-α Receptor Fusion Protein Prevents Experimental Auto-Immune Encephalomyelitis and Demyelination in Lewis Rats: an Overview, The Journal of Neuroimmunology, 72:163-168 (1997).
Kohno et al., A Second Tumor Necrosis Factor Receptor Gene Product Can Shed a Naturally Occurring Tumor Necrosis Factor Inhibitor, Proc. Natl. Acad. Sci. USA, 87:8331-8335 (1990).
Kruse et al., Conversion of Human Interleukin-4 Into a High Affinity Antagonist by a Single Amino Acid Replacement, The EMBO Journal, 11:3237-3244 (1992).
Kull et al., Cellular Receptor for $^{125}$I-Labeled Tumor Necrosis Factor: Specific Binding, Affinity Labeling, and Relationship to Sensitivity, Proc. Natl. Acad. Sci. USA, 82:5756-5760 (1985).
Landolfi, A Chimeric IL-2/Ig Molecule Possesses the Functional Activity of Both Proteins, J. Immunol., 146:915-919 (1991).
Langner et al., Structural and Functional Analysis of a TNF Receptor-Immunoglobulin Fusion Protein, New Advances on Cytokines, 349-354 (1992).
Leberthon et al., Enhanced Tumor Uptake of Macromolecules Induced by a Novel Vasoactive Interleukin 2 Immunoconjugate, Cancer Research, 51:2694-2698 (1991).
Lesslauer et al., Recombinant Soluble Tumor Necrosis Factor Receptor Proteins Protect Mice From Lipopolysaccharide-Induced Lethality, Eur. J. Immunol., 21:2883-2886 (1991).
Liabakk et al., A Rapid and Sensitive. Immunoassay for Tumor Necrosis Factor Using Magnetic Monodisperse Polymer Particles, Journal of Immunological Methods, 134:253-259 (1990).
Loetscher et al., Efficacy of a Chimeric TNFR-IgG Fusion Protein to Inhibit TNF Activity in Animal Models of Septic Shock, Endotoxin Research Series, 2:455-462 (1993).
Loetscher et al., Two distinct human TNF receptors: purification, molecular cloning and expression, in Tumor Necrosis Factor: Structure-Function Relationship and Clinical Application, (3$^{rd}$ International Conference.
Maliszewski et al., Cytokine Receptors and B Cell Functions: Recombinant Soluble Receptors Specifically Inhibit IL-1 and IL-4 Induced. Cell Activities in Vitro, J. Immunol., 144:3028-3033(1990).
Mohler et al., Soluble Tumor Necrosis Factor (TNF) Receptors are Effective Therapeutic Agents in Lethal Endotoxemia and Function Simultaneously as Both TNF Carriers and TNF Antagonists, J. Immunol., 151:1548-1561 (1993).
Mori et al., Attenuation of Collagen-Induced Arthritis in 55-kDa TNF Receptor Type 1 (TNFR1)-IgG1-Treated and TNFR1-Deficient Mice, J. Immunol., 157:3178-3182 (1996).
Morrissey et al., Molecular Cloning of the cDNA for Tissue Factor, the Cellular Receptor for the Initiation of the Coagulation Protease Cascade, Cell: 50:129-135 (1987).
Morrison, in Vitro Antibodies: Strategies for Production and Application, Annu. Rev. Immunol., 10:239-265 (1992).
Mosley et al., The Murine Interleukin-4 Receptor: Molecular Cloning and Characterization of Secreted and Membrane Bound Forms, Cell: 59:335-348 (1989).
Nophar et al., Soluble Forms of Tumor Necrosis Factor Receptors (TNF-Rs). The cDNA for the Type I TNF-R, Cloned Using Amino Acid Sequence Data of its Soluble Form, Encodes Both the Cell Surface and a Soluble Form of the Receptor, The EMBO Journal, 9:3269-3278 (1990).
Novotny et al., A Soluble, Single-Chain T-Cell Receptor Fragment Endowed With Antigen-Combining Properties, Proc. Natl. Acad. Sci. USA, 88:8646-8650 (1991).
Okayama et al., High-Efficiency Cloning of Full-Length cDNA, Molecular and Cellular Biology, 2:161-170 (1982).
Okayama et al., A cDNA Cloning Vector That Permits Expression of cDNA Inserts in Mammalian Cells, Molecular and Cellular Biology,..3:280-289 (1983).
Old, Tumor Necrosis Factor, 2nd Intl Conference on Tumor Necrosis Factor & Related Cytokines, Napa, CA, 1-30 (1989).
Paborsky et al., Purification of Recombinant Human Tissue Factor, Biochemistry, 28:8072-8077 (1989).
Parrillo, Pathogenetic Mechanisms of Septic Shock, New Eng. J. Med., 328:1471-1477 (1993).
Peetre et al., A Tumor Necrosis Factor Binding Protein is Present in Human Biological Fluids, Eur. J. Haematol. 41:414-419 (1988).
Pennica et al., Human Tumour Necrosis Factor: Precursor Structure, Expression and Homology to Lymphotoxin, Nature, 312:724-729 (1984).
Peppel et al., Chimaeric TNF-Receptor—IgG Molecule Acts As Soluble Inhibitor of TNF Mediated Cytotoxicity, J. Cell. Biochem., Supp. 15F:439 (1991).
Piguet et al., Evolution of Collagen Arthritis in Mice is Arrested by Treatment With Anti-Tumor Necrosis (TNF) Antibody or a Recombinant Soluble TNF Receptor, Immunology, 77 (4):510-514 (1992).
Redfield et al., Secondary Structure and Topology of Human Interleukin 4 in Solution, Biochemistry, 30:11029-11035 (1991).
Rubin, Binding Receptor Characters Zako and Expression, and IntracellularEvents. 2nd Intl Conference on Tumor Necrosis Factor & Related Cytokines, Napa, CA, 94-96 (1989).
Ruddle et al., An Antibody to Lymphotoxin and Tumor Necrosis Factor Prevents Transfer of Experimental Allergic Encephalomyelitis, J. Exp. Med., 172:1193-1200 (1990).
Rutka et al., The Effects of Human Recombinant Tumor Necrosis Factor on Glioma-Derived Cell Lines: Cellular Proliferation, Cytotoxicity, Morphological and Radioreceptor Studies, Int. J. Cancer., 41:573-582 (1988).
Saxne et al.; Detection of Tumor Necrosis Factor α But Not Tumor Necrosis Factor β in Rheumatoid Arthritis Synovial Fluid and Serum, Arthritis & Rheumatism, 31:1041-1045 (1988).
Scallon of al., Functional Comparisons of Different Tumour Necrosis Factor Receptor/IgG Fusion Proteins, Cytokine, 7:759-770 (1995).
Scarpati et al., Human Tissue Factor, cDNA Sequence and Chromosome Localization of the Gene, Biochemistry, 26:5234-5238 (1987).
Schleiffenbaum et al., The Tumor Necrosis Factor Receptor and Human Neutrophil Function, J. Clin. Invest., 86:184-195 (1990).
Schnee et al., Construction and Expression of a Recombinant Antibody-Targeted Plasminogen Activator, Proc. Natl. Acad. Sci. USA, 84:6904-6908 (1987).
Seckinger et al., A Human Inhibitor of Tumor Necrosis Factor α, J. Exp. Med. 167:1511-1516 (1988).
Shalaby et al., Receptor Binding and Activation of Polmorphonuclear Neutrophils by Tumor Necrosis Factor-Alpha, Joumal of Leukocyte Biology, 41:196-204 (1987).
Shalaby et al., Binding and Regulation of Cellular Function by Monoclonal antibodies Against Human Tumor Necrosis Factor Receptors, J. Exp. Med. 172: 1517-1520 (1990).
Sheehan et al., Generation and Characterization of Hamster Monoclonal Antibodies That Neutralize Murine Tumor Necrosis Factors, Journal of Immunology, 142:3884-3893 (1989).
Shin et al., Expression and Characterization of an Antibody Binding Specificity Joined to Insulin-Like Growth Factor 1: Potential Applications for Cellular Targeting, Proc. Natl. Acad. Sci., 87:5322-5326 (1990).
Sims et al., cDNA Expression Cloning of the IL-1 Receptor, a Member of the Immunoglobulin Superfamily, Science, 241:585-589 (1988).
Sims et al., Cloning the Interleukin 1 Receptor From Human T Cells, Proc. Natl. Acad. Sci., 86:8946-8950 (1989).
Smith et al.; The Active Form of Tumor Necrosis Factor Is a Trimer, J. Biol. Chem., 262:6951-6954 (1987).
Smith et al., Blocking of HIV-1 Infectivity by a Soluble, Secreted Form of the CD4 Antigen, Science, 238:1704-1707 (1987).
Smith et al., Multimeric Structure of the Tumor Necrosis Factor Receptor of HeLa Cells, J. Biol. Chem., 264:14646-14652 (1989).
Spicer et al., Isolation of cDNA Clones Coding for Human Tissue Factor: Primary Structure of the Protein and cDNA, Proc. Natl. Acad. Sci., 84:5148-5152 (1987).
Staines et al., Collagen Arthritis-What Can It Teach Us?, British Journal of Rheumatology, 33:798-807 (1994).
Strader et al., Structural Basis of β-Adrenergic Receptor Function, The FASEB Journal, 3:1825-1832 (1989).
Suggs et al., Use of Synthetic Oligonucleotides as Hybridization Probes: Isolation of Cloned cDNA Sequences for Human $\beta_2$-Microglobulin, Proc. Natl. Acad. Sci. U.S.A., 78:6613-6617 (1981).
Tauber et al:, Toxicity in Neuronal Cells Caused by Cererospinal Fluid fFom Pneumococcal and Gram-Negative Meningitis, The Journal of Infectious Diseases, 166:1045-1050 (1992).

Thoma et al., Identification of a 60-kD Tumor Necosis Factor (TNF) Receptor as the Major Signal Transducing Component in TNF Responses, J. Exp. Med. 172: 1019-23 (1990).
Tsujimoto et al., Characterization and Affinity Crosslinking of Receptors for Tumor Necrosis Factor on Human Cells, Archives of Biochemistry and Biophysics, 249:563-568 (1986).
Tsujimoto et al, Interferon-γ Enhances Expression of Cellular Receptors for Tumor Necrosis Factor, J. Immunol., 136:2441-2444 (1986).
Tsujimoto et al, Tumor necrosis factor: specific binding and internalization in sensitive and resistant cells, Proc. Natl. Acad. Sci. 82: 7626-30 (1985).
Ulich et al., Intratracheal Administration of Endotoxin and Cytokines, Clinical Immunology & Immunopathology., 72:137-140 (1994).
Unglaub et al., Downregulation of Tumor Necrosis Factor (TNF) Sensitivity Via Modulation of TNF Binding Capacity by Protein Kinase C Activators, J. Exp. Med. 166:1788-1797 (1987).
Van Der Poll et al., Pretreatment with a 55-kDa Tumor Necrosis Factor Receptor—Immunoglobulin Fusion Protein Attenuates Activation of Coagulation, but not of Fibrinolysis, during Lethal Bacteremia in Baboons, The Journal of Infectious Diseases., 176:296-299 (1997).
Van Zee et al., Protection Against Lethal *Escherichia coil* Bacteremia in Baboons (*Papio anubis*) by Pretreatment With a 55-kDa TNF Receptor (CD120a)-Ig Fusion Protein, Ro 45-2081, J. Immunol., 156:2221-2230 (1996).
Wallach et al., Soluble and Cell Surface Receptors for Tumor Necrosis Factor, Progress, Inflammation Research & Therapy, 51-57 (1991).
Wallach et al., Cell surface and soluble TNF receptors, in Tumor Necrosis Factor: Structure-Function Relationship and Clinical Application, (3rd International Conference on Tumor Necrosis Factor and Related Cytokines, Makuhari, Chiba, Nov. 21-25, 1990), Osawaand Bonavida, eds., Basel, Karger, pp. 47-57 (1992).
Wilks, The CD4 Receptor: Post Binding Events, Conformational Change and the Second Site, Molec. Aspects Med., 12:255-265 (1991).
Yamasaki et al., Cloning and Expression of the Human Interleukin-6 (BSF-2/IFNβ 2) Receptor, Science, 241:825-282 (1988).
Yonehara et al., A Cell-Killing Monoclonal Antibody (Anti-Fas) to a Cell Surface Antigen Co-Downregulated With the Receptor of Tumor Necrosis Factor, J. Exp. Med., 169:1747-1765 (1989).
Yoshie et al., Binding and Crosslinking of $^{125}$I-Labeled Recombinant Human Tumor Necrosis Factor to Cell Surface Receptors, J. Biochem., 100: 531-541(1986).
Brower et al. Roche's RA Drug Crippled, Nature Biotechnology, 15:1325 (1997).
Nesbitt, et al., "Mechanism of Action of Certolizumab Pegol (CDP870): In Vitro Comparison with Other Anti-tumor Necrosis Factor a Agents", *Inflamm Bowel Dis*, 13: 1323-1332 (Nov. 2007).
Barone et al., Comparative Analysis of the Ability of Etanercept and Infliximab to Lyse TNF-Expressing Cells in a Complement Dependent Fashion. Arthritis Rheum., 42(9) supplement, Sep. 1999 (S90).
Bringman et al., Monoclonal antibodies to human tumor necrosis factors alpha and beta: application for affinity purification, immunoassays, and as structural probes. *Hybridoma*, 6(5):489-507 (1987).
Byrn et al., Biological properties of a CD4 immunoadhesin. *Nature*, 344:667-70 (1990).
Capon et al., Designing CD4 immunoadhesins for AIDS therapy. *Nature*, 337:525-31 (1989).
Cosman et al., A new cytokine receptor superfamily. *Trends Biochem. Sci.* 15:265-70 (1990).
Deen et al. A soluble form of CD4 (T4) protein inhibits AIDS virus infection. *Nature*, 331(6151): 82-4 (1988).
Dembic et al., Two Human TNF receptors have similar extracellular, but distinct intracellular, domain sequences. *Cytokine* 2: 231-237, 1990).
Berke, Functions and mechanisms of lysis induced by cytotoxic T lymphocytes and natural killer cells. Fundamental Immunology, 2nd Edition, Paul, ed., Raven Press, New York, pp. 735-64 (1989).
Heller et al., Complementary DNA cloning of a receptor for tumor necrosis factor and demonstration of a shed form of the receptor. *Proc. Natl. Acad. Sci USA*, 87:6151-5 (1990).
Irwin et al, Affinity precipitation methods, Chapter 22, Methods in Molecular Biology, 59: 217-38 (1996).
Khare et al, Mechanisms of cell death induced by tumor necrosis factor antagonists. Poster 715 presented at the Annual Meeting of the Society for Investigative Dermatology (SID), May 3-5, 2006, Philadelphia, PA.
Kohno et al., Adalimumab and Infliximab bind to Fc-receptor and C1q and generate immunoprecipitation: A different mechanism from Etanercept. Presentation 1495, Poster 271, presented at the American College of Rheumatology Annual Meeting, Nov. 13-17, 2005, San Diego, CA.
Larsson et al., Affinity precipitation of enzymes. *FEBS Lett.* 98(2):333-8 (1979).
Mohler et al., Soluble tumor necrosis factor (TNF) receptors are effective therapeutic agents in lethal endotoxemia and function simultaneously as both TNF carriers and TNF antagonists. *J. Immunol.*, 151:1548-61 (1993).
Sell, Immunology, Immunopathology and Immunity, 4th Edition, Elsevier Science Publishing Co., New York, 1987, at pp. 85-91.
Smith et al., Multimeric structure of the tumor necrosis factor receptor of HeLa cells. *J. Biol. Chem.* 262:6951-4 (1987).
Smith et al., A receptor for tumor necrosis factor defines an unusual family of cellular and viral proteins. *Science*, 248:1019-23 (1990).
Traunecker et al., Highly efficient neutralization of HIV with recombinant CD4-immunoglobulin molecules. *Nature*, 339:68-70 (1989).
Williams et al., Identification of a ligand for the c-kit proto-oncogene. *Cell*, 63: 167-74 (1990).
Wingfield et al., Tumour necorsis factor is a compact trimer. *FEBS Lett.* 211: 179-84 (1987).
Evans et al., Protective effect of 55- but not 75-kD soluble tumor necrosis factor receptor-immunoglobulin G fusion proteins in an animal model of gram-negative sepsis. *J. Exp. Med.* 180: 2173-9 (1994).
Exhibit A: Memorandum by D. Urdal to S. Gillis, M. Kranda, and P. Grassam, dated Oct. 27, 1989.
Exhibit B: Correspondence from D. Urdal to L. Lauffer dated Feb. 26, 1990.
Exhibit C: Lab Notebook of E. Jeffrey, pages dated May 1990 through Jan. 1991.
Exhibit D: Correspondence from L. Lauffer to D. Urdal, dated May 21, 1990.
Exhibit E: Meeting minutes, Immunex employee (author unknown) to file, dated Jun. 25, 1990.
Exhibit F: Lab notebook of Terri Davis, pages dated Jul. 11, 1990.
Exhibit G: Letter from M. Deeley to L. Lauffer, dated Jul. 20, 1990.
Exhibit H: Meeting minutes, Immunex employee (author unknown) to file, dated Jul. 23, 1990.
Exhibit I: Correspondence from Drs. Seiler and Zeittmeissl to D. Gillis, dated Aug. 8, 1990.
Exhibit J (J1-J21): Declaration of Bruce A. Beutler, Karsten Peppel, and David F. Crawford submitted to the USPTO on Jul. 16, 1993 during the prosecution of U.S. Appl. No. 07/862,495, filed Apr. 2, 1992 (issued as US 5,447,851 naming inventors B. Beutler, K. Peppel, and D. Crawford), including exhibits J-1-J21, which were submitted with the declaration.
Exhibit K: Confirmation page from D. Urdal to P. Oquendo, dated Oct. 4, 1990.
Letter from J. Thomas to L. Lauffer dated Dec. 10, 1990.
Memo from J. Thomas to P. Baum, D. Cosman, M. Deeley, R. Goodwin, S. Gillis, H. Sassenfeld, and D. Urdal, dated Dec. 17, 1990, conveying attached facsimile received Dec. 13, 1990 from L. Lauffer to J. Thomas.
Declaration of Taruna Arora under 37 C.F.R. § 1.132 plus Exhibits A-D dated Dec. 16, 2010, filed in sister case U.S. Appl. No. 08/444,790 (which was filed on May 19, 1995, inventors M. Brockhaus, Z. Dembic, R. Gentz, W. Lesslauer, H. Loetscher, E. Schlaeger, hereinafter "U.S. Appl. No. 08/444,790").
Arora et al., "Differences in Binding and Effector Functions Between Classes of TNF Antagonists," Cytokine 45: 124-131 (2009).

Arruffo et al., "CD44 Is the Principal Cell Surface Receptor for Hyaluronate," Cell 61: 1303-1313 (1990).
Brennan et al., "Inhibitory Effect of TNFα Antibodies on Synovial Cell Interleukin-1 Production in Rheumatoid Arthritis," Lancet 2(8657): 244-247 (1989).
Chan et al., "A Domain in TNF Receptors That Mediates Ligand-Independent Receptor Assembly and Signaling," Science 288: 2351-2354 (2000).
Engelmann et al., "Two Tumor Necrosis Factor-binding Proteins Purified from Human Urine," J. Biol. Chem. 265(3): 1531-36 (1990).
Mitoma et al., "Mechanisms for Cytotoxic Effects of Anti-Tumor Necrosis Factor Agents on Transmembrane Tumor Necrosis Factor a-Expressing Cells," Arthr. & Rheum. 58(5): 1248-1257 (2008).
Novick et al., "Soluble Cytokine Receptors Are Present in Normal Human Urine," J. Exp. Med., 170: 1409-1414 (1989).
Shalaby et al., The Involvement of Human Tumor Necrosis Factors-α and -β in the Mixed Lymphocyte Reaction, J. Immunol. 141: 499-503 (1988).
Smith and Baglioni, "Multimeric Structure of the Tumor Necrosis Factor Receptor of HeLa Cells," J. Biol. Chem. 264: 14646-14652 (1989).
Strand et al., "Biologic Therapies in Rheumatology: Lessons Learned, Future Directions," Nature Rev. 6: 75-92 (2007).
Strangfeld et al., "Risk of Herpes Zoster in Patients with Rheumatoid Arthritis Treated with AntiTNF-α Agents," JAMA 301(7): 737-744 (2009).
Wallis et al., "Reactivation of Latent Granulomatous Infections by Infliximab," Clin. Inf. Dis. 41(Suppl 2): S1-S5 (2005).
Wallis et al., "Granulomatous Infectious Diseases Associated with Tumor Necrosis Factor Antagonists," Clin. Inf. Dis. 38: 1261-1265 (2004).
Watson et al., "A Homing Receptor—IgG Chimera as a Probe for Adhesive Ligands of Lymph Node High Endothelial Venules," J. Cell Biol. 110: 2221-2229 (1990).
Winzor et al., "Evaluation of Equilibrium Constants from Precipitin Curves: Interaction of α-Crystallin with an Elicited Monoclonal Antibody," Arch. Biochem. Biophys. 268(1): 221-226 (1989).
Furst et al. "Tumor Necrosis Factor Antagonists: Different Kinetics and/or Mechanisms of Action may Explain Differences in the Risk for Developing Granulomatous Infection," Semin. Arthritis Rheum. 36(3):159-67. (2006).
United States Adopted Names (USAN) Council Report, *Clin. Pharm. & Ther.*, vol. 66, No. 2, Aug. 1999, p. 209.
Feldmann et al., "Cytokine production in the rheumatoid joint: implications for treatment," Ann. Rheum. Dis. 49: 480-486 (1990).
Hoogenboom et al., "Construction and Expression of Antibody-Tumor Necrosis Factor Fusion Proteins," Molecular Immunol. 28(9): 1027-1037 (1991).
US 6,224,867, 05/2001, Smith et al. (withdrawn)

* cited by examiner

FIGURE 1A

```
-185 GAATTCGGGGGGGTTCAAGATCACTGGGACCAGGCCGTGATCTCTATGCCCGAGTCTCAA
-125 CCCTCAACTGTCACCCCAAGGCACTTGGGACGTCCTGGACAGACCGAGTCCCGGGAAGCC
 -65 CCAGCACTGCCGCTGCCACACTGCCCTGAGCCCAAATGGGGGAGTGAGAGGCCATAGCTG
          -28.         .         .         .         .         .
 -30      MetGlyLeuSerThrValProAspLeuLeuLeuProLeuValLeuLeuGluLeu
  -5 TCTGGCATGGGCCTCTCCACCGTGCCTGACCTGCTGCTGCCGCTGGTGCTCCTGGAGCTG
                .         .         .   +1    .         .         .
 -10 LeuValGlyIleTyrProSerGlyValIleGlyLeuValProHisLeuGlyAspArgGlu
  55 TTGGTGGGAATATACCCCTCAGGGGTTATTGGACTGGTCCCTCACCTAGGGGACAGGGAG
                .         .         .         .     ***   .         .
  10 LysArgAspSerValCysProGlnGlyLysTyrIleHisProGlnAsnAsnSerIleCys
 115 AAGAGAGATAGTGTGTGTCCCCAAGGAAAATATATCCACCCTCAAAATAATTCGATTTGC
                .         .         .         .         .         .
  30 CysThrLysCysHisLysGlyThrTyrLeuTyrAsnAspCysProGlyProGlyGlnAsp
 175 TGTACCAAGTGCCACAAAGGAACCTACTTGTACAATGACTGTCCAGGCCCGGGGCAGGAT
                .         .         .         .         .         .
  50 ThrAspCysArgGluCysGluSerGlySerPheThrAlaSerGluAsnHisLeuArgHis
 235 ACGGACTGCAGGGAGTGTGAGAGCGGCTCCTTCACCGCTTCAGAAAACCACCTCAGACAC
                .         .         .         .         .         .
  70 CysLeuSerCysSerLysCysArgLysGluMetGlyGlnValGluIleSerSerCysThr
 295 TGCCTCAGCTGCTCCAAATGCCGAAAGGAAATGGGTCAGGTGGAGATCTCTTCTTGCACA
                .         .         .         .         .         .
  90 ValAspArgAspThrValCysGlyCysArgLysAsnGlnTyrArgHisTyrTrpSerGlu
 355 GTGGACCGGGACACCGTGTGTGGCTGCAGGAAGAACCAGTACCGGCATTATTGGAGTGAA
```

FIGURE 1B

```
                  .         ***         .         ***.          .          .
110  AsnLeuPheGlnCysPheAsnCysSerLeuCysLeuAsnGlyThrValHisLeuSerCys
415  AACCTTTTCCAGTGCTTCAATTGCAGCCTCTGCCTCAATGGGACCGTGCACCTCTCCTGC
                  .         .         .         .          .          .
130  GlnGluLysGlnAsnThrValCysThrCysHisAlaGlyPhePheLeuArgGluAsnGlu
475  CAGGAGAAACAGAACACCGTGTGCACCTGCCATGCAGGTTTCTTTCTAAGAGAAAACGAG
                  .         .         .         .          .          .
150  CysValSerCysSerAsnCysLysLysSerLeuGluCysThrLysLeuCysLeuProGln
535  TGTGTCTCCTGTAGTAACTGTAAGAAAAGCCTGGAGTGCACGAAGTTGTGCCTACCCCAG
                  .         .         .         .          .          .
170  IleGluAsnValLysGlyThrGluAspSerGlyThrThrValLeuLeuProLeuValIle
595  ATTGAGAATGTTAAGGGCACTGAGGACTCAGGCACCACAGTGCTGTTGCCCCTGGTCATT
                  .         .         .         .          .          .
190  PhePheGlyLeuCysLeuLeuSerLeuLeuPheIleGlyLeuMetTyrArgTyrGlnArg
655  TTCTTTGGTCTTTGCCTTTTATCCCTCCTCTTCATTGGTTTAATGTATCGCTACCAACGG
                  .         .         .         .          .          .
210  TrpLysSerLysLeuTyrSerIleValCysGlyLysSerThrProGluLysGluGlyGlu
715  TGGAAGTCCAAGCTCTACTCCATTGTTTGTGGGAAATCGACACCTGAAAAAGACGGGGAG
                  .         .         .    ***    .          .          .
230  LeuGluGlyThrThrThrLysProLeuAlaProAsnProSerPheSerProThrProGly
775  CTTGAAGGAACTACTACTAAGCCCCTGGCCCCAAACCCAAGCTTCAGTCCCACTCCAGGC
```

FIGURE 1C

```
                .         .         .         .         .         .
 250  PheThrProThrLeuGlyPheSerProValProSerSerThrPheThrSerSerSerThr
 835  TTCACCCCCACCCTGGGCTTCAGTCCCGTGCCCAGTTCCACCTTCACCTCCAGCTCCACC
                .         .         .         .         .         .
 270  TyrThrProGlyAspCysProAsnPheAlaAlaProArgArgGluValAlaProProTyr
 895  TATACCCCCGGTGACTGTCCCAACTTTGCGGCTCCCCGCAGAGAGGTGGCACCACCCTAT
                .         .         .         .         .         .
 290  GlnGlyAlaAspProIleLeuAlaThrAlaLeuAlaSerAspProIleProAsnProLeu
 955  CAGGGGGCTGACCCCATCCTTGCGACAGCCCTCGCCTCCGACCCCATCCCCAACCCCCTT
                .         .         .         .         .         .
 310  GlnLysTrpGluAspSerAlaHisLysProGlnSerLeuAspThrAspAspProAlaThr
1015  CAGAAGTGGGAGGACAGCGCCCACAAGCCACAGAGCCTAGACACTGATGACCCCGCGACG
                .         .         .         .         .         .
 330  LeuTyrAlaValValGluAsnValProProLeuArgTrpLysGluPheValArgArgLeu
1075  CTGTACGCCGTGGTGGAGAACGTGCCCCCGTTGCGCTGGAAGGAATTCGTGCGGCGCCTA
                .         .         .         .         .         .
 350  GlyLeuSerAspHisGluIleAspArgLeuGluLeuGlnAsnGlyArgCysLeuArgGlu
1135  GGGCTGAGCGACCACGAGATCGATCGGCTGGAGCTGCAGAACGGGCGCTGCCTGCGCGAG
                .         .         .         .         .         .
 370  AlaGlnTyrSerMetLeuAlaThrTrpArgArgArgThrProArgArgGluAlaThrLeu
1195  GCGCAATACAGCATGCTGGCGACCTGGAGGCGGCGCACGCCGCGGCGCGAGGCCACGCTG
                .         .         .         .         .         .
 390  GluLeuLeuGlyArgValLeuArgAspMetAspLeuLeuGlyCysLeuGluAspIleGlu
1255  GAGCTGCTGGGACGCGTGCTCCGCGACATGGACCTGCTGGGCTGCCTGGAGGACATCGAG
```

FIGURE 1D

```
                .         .         .         .         .
  410 GluAlaLeuCysGlyProAlaAlaLeuProProAlaProSerLeuLeuArg
 1315 GAGGCGCTTTGCGGCCCCGCCCCTCCCCGCGCCCCAGTCTTCTCAGATGAGGCTGC
 1375 GCCCCCTGCGGGCAGCTCTAAGGACCGTCCTGCGAGATCGCCTTCCAACCCCACTTTTTTC
 1435 TGGAAAGGAGGGGTCCTGCAGGGGCAAGCAGGAGCTAGCAGCCGCCTACTTGGTGCTAAC
 1495 CCCTCGATGTACATAGCTTTTCTCAGCTGCCTGCGCGCCGCCGACAGTCAGCGCTGTGCG
 1555 CGCGGAGAGAGGTGCGCCGTGGGCTCAAGAGCCTGAGTGGGTGGTTTGCGAGGATGAGGG
 1615 ACGCTATGCCTCATGCCCGTTTTGGGTGTCCTCACCAGCAAGGCTGCTCGGGGGCCCCTG
 1675 GTTCGTCCCTGAGCCTTTTTTCACAGTGCATAAGCAGTTTTTTTTTGTTTTTGTTTTT
 1735 GTTTTGTTTTTTAAATCAATCATGTTACACTAATAGAAACTTGGCACTCCTGTGCCCTCTG
 1795 CCTGGACAAGCACACATAGCAAGCTGAACTGTCCTAAGGCAGGGGCGAGCACGGAACAATGG
 1855 GGCCTTCAGCTGGAGCTGTGGACTTTTGTACATACACTAAAATTCTGAAGTTAAAAAAAA
 1915 AACCCGAATTC
```

Figure 2A

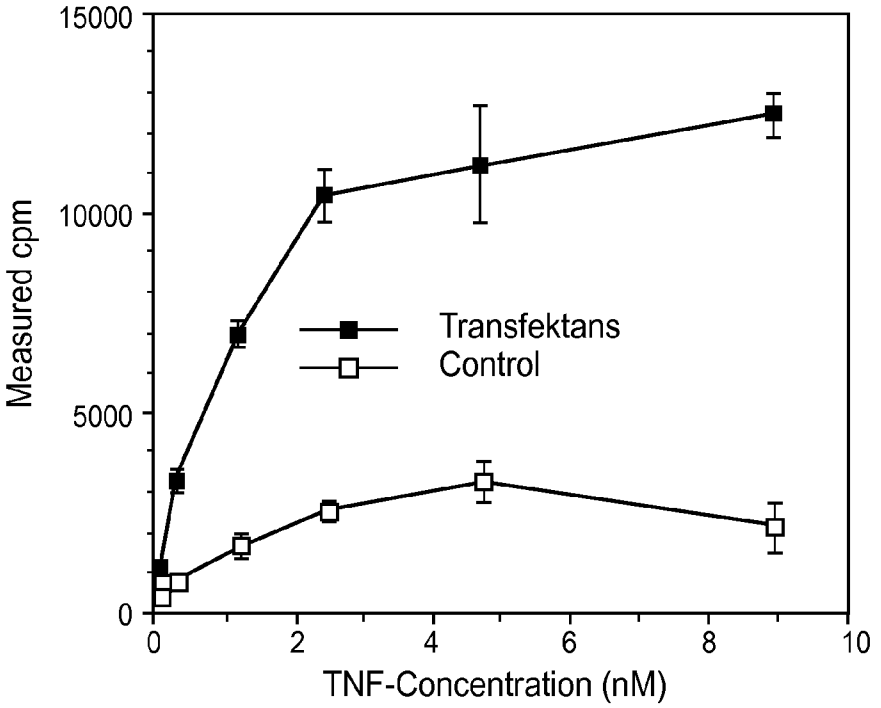



Figure 2B

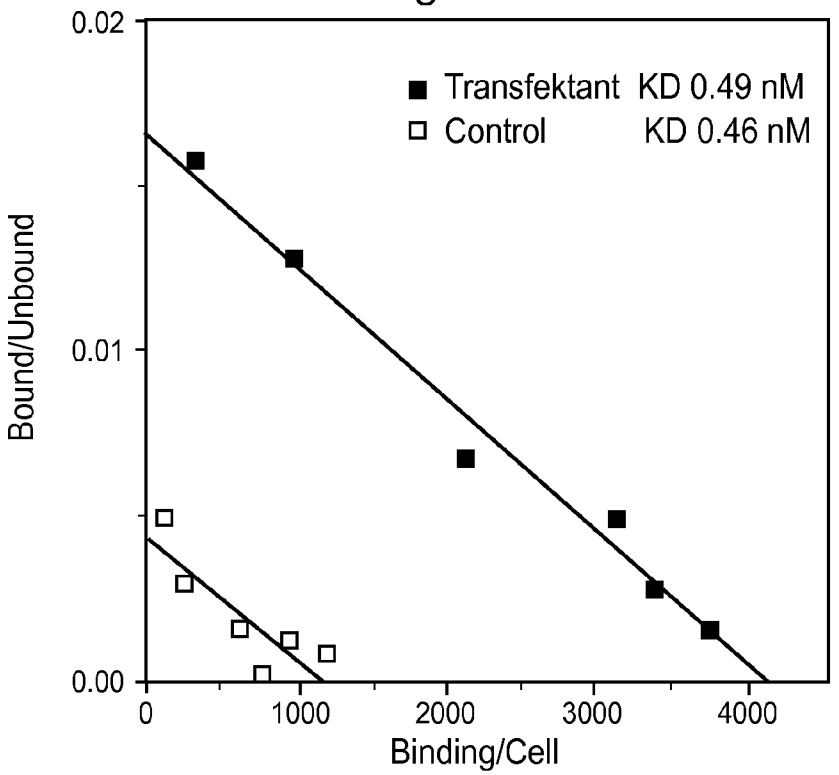


Figure 3

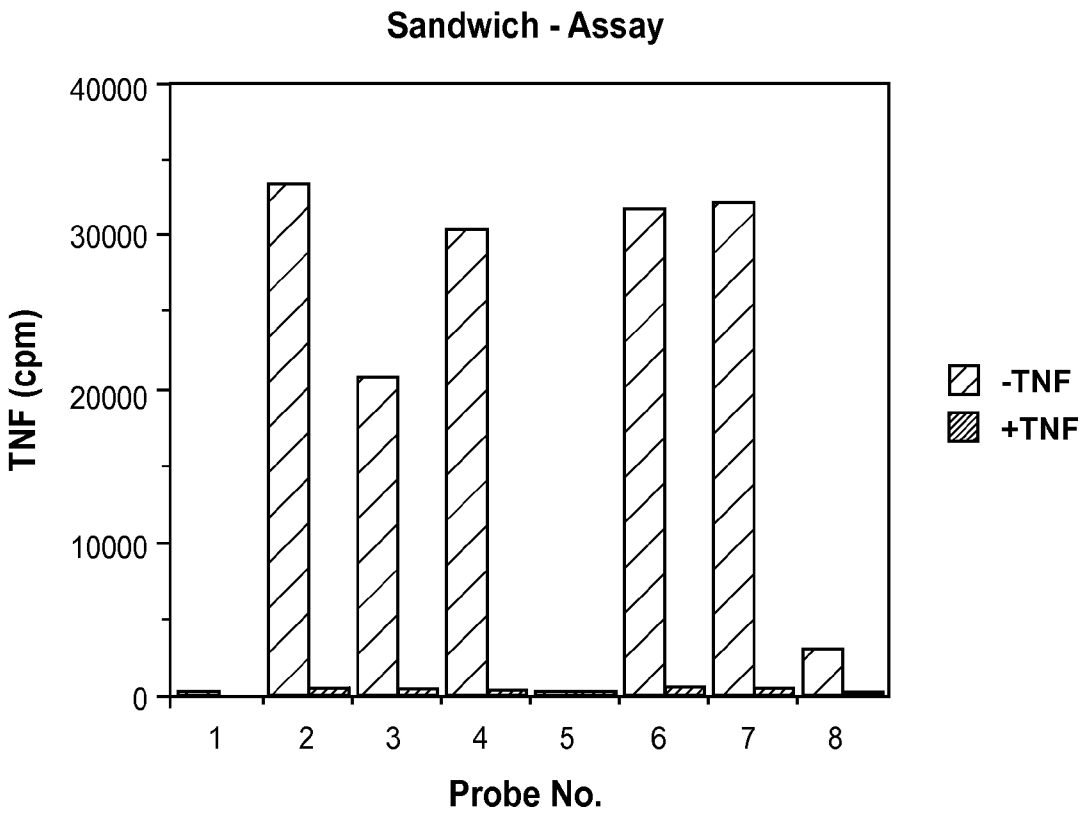

Sandwich - Assay
TNF (cpm)
40000
30000
20000
10000
0
1
2
3
4
5
6
7
8
Probe No.
-TNF
+TNF

FIGURE 4A

```
                .         .         .         .         .         .
  1 SerAspSerValCysAspSerCysGluAspSerThrTyrThrGlnLeuTrpAsnTrpVal
  1 TCGGACTCCGTGTGTGACTCCTGTGAGGACAGCACATACACCCAGCTCTGGAACTGGGTT
                .         .         .         .         .         .
 21 ProGluCysLeuSerCysGlySerArgCysSerSerAspGlnValGluThrGlnAlaCys
 61 CCCGAGTGCTTGAGCTGTGGCTCCCGCTGTAGCTCTGACCAGGTGGAAACTCAAGCCTGC
                .         .         .         .         .         .
 41 ThrArgGluGlnAsnArgIleCysThrCysArgProGlyTrpTyrCysAlaLeuSerLys
121 ACTCGGGAACAGAACCGCATCTGCACCTGCAGGCCCGGCTGGTACTGCGCGCTGAGCAAG
                .         .         .         .         .         .
 61 GlnGluGlyCysArgLeuCysAlaProLeuProLysCysArgProGlyPheGlyValAla
181 CAGGAGGGGTGCCGGCTGTGCGCGCCGCTGCCGAAGTGCCGCCCGGGCTTCGGCGTGGCC
                .         .         .         .         .         .
 81 ArgProGlyThrGluThrSerAspValValCysLysProCysAlaProGlyThrPheSer
241 AGACCAGGAACTGAAACATCAGACGTGGTGTGCAAGCCCTGTGCCCCGGGGACGTTCTCC
                .         .         .         .         .         .
101 AsnThrThrSerSerThrAspIleCysArgProHisGlnIleCysAsnValValAlaIle
301 AACACGACTTCATCCACGGATATTTGCAGGCCCCACCAGATCTGTAACGTGGTGGCCATC
                .         .         .         .         .         .
121 ProGlyAsnAlaSerArgAspAlaValCysThrSerThrSerProThrArgSerMetAla
361 CCTGGGAATGCAAGCAGGGATGCAGTCTGCACGTCCACGTCCCCCACCCGGAGTATGGCC
                .         .         .         .         .         .
141 ProGlyAlaValHisLeuProGlnProValSerThrArgSerGlnHisThrGlnProSer
421 CCAGGGGCAGTACACTTACCCCAGCCAGTGTCCACACGATCCCAACACACGCAGCCAAGT
```

FIGURE 4B

```
                .         .         .         .         .         .
161 ProGluProSerThrAlaProSerThrSerPheLeuLeuProMetGlyProSerProPro
481 CCAGAACCCAGCACTGCTCCAAGCACCTCCTTCCTGCTCCCAATGGGCCCCAGCCCCCCA
                .         .         .         .         .         .
181 AlaGluGlySerThrGlyAspPheAlaLeuProValGlyLeuIleValGlyValThrAla
541 GCTGAAGGGAGCACTGGCGACTTCGCTCTTCCAGTTGGACTGATTGTGGGTGTGACAGCC
                .         .         .         .         .         .
201 LeuGlyLeuLeuIleIleGlyValValAsnCysValIleMetThrGlnValLysLysLys
601 TTGGGTCTACTAATAATAGGAGTGGTGAACTGTGTCATCATGACCCAGGTGAAAAAGAAG
                .         .         .         .         .         .
221 ProLeuCysLeuGlnArgGluAlaLysValProHisLeuProAlaAspLysAlaArgGly
661 CCCTTGTGCCTGCAGAGAGAAGCCAAGGTGCCTCACTTGCCTGCCGATAAGGCCCGGGGT
                .         .         .         .         .         .
241 ThrGlnGlyProGluGlnGlnHisLeuLeuIleThrAlaProSerSerSerSerSerSer
721 ACACAGGGCCCCGAGCAGCAGCACCTGCTGATCACAGCGCCGAGCTCCAGCAGCAGCTCC
                .         .         .         .         .         .
261 LeuGluSerSerAlaSerAlaLeuAspArgArgAlaProThrArgAsnGlnProGlnAla
781 CTGGAGAGCTCGGCCAGTGCGTTGGACAGAAGGGCGCCCACTCGGAACCAGCCACAGGCA
                .         .         .         .         .         .
281 ProGlyValGluAlaSerGlyAlaGlyGluAlaArgAlaSerThrGlySerSerAlaAsp
841 CCAGGCGTGGAGGCCAGTGGGGCCGGGGAGGCCCGGGCCAGCACCGGGAGCTCAGCAGAT
                .         .         .         .         .         .
301 SerSerProGlyGlyHisGlyThrGlnValAsnValThrCysIleValAsnValCysSer
901 TCTTCCCCTGGTGGCCATGGGACCCAGGTCAATGTCACCTGCATCGTGAACGTCTGTAGC
```

FIGURE 4C

```
                .         .         .         .         .         .
 321  SerSerAspHisSerSerGlnCysSerSerGlnAlaSerSerThrMetGlyAspThrAsp
 961  AGCTCTGACCACAGCTCACAGTGCTCCTCCCAAGCCAGCTCCACAATGGGAGACACAGAT
                .         .         .         .         .         .
 341  SerSerProSerGluSerProLysAspGluGlnValProPheSerLysGluGluCysAla
1021  TCCAGCCCCTCGGAGTCCCCGAAGGACGAGCAGGTCCCCTTCTCCAAGGAGGAATGTGCC
                .         .         .         .         .         .
 361  PheArgSerGlnLeuGluThrProGluThrLeuLeuGlySerThrGluGluLysProLeu
1081  TTTCGGTCACAGCTGGAGACGCCAGAGACCCTGCTGGGGAGCACCGAAGAGAAGCCCCTG
                .         .         .         .         .         .
 381  ProLeuGlyValProAspAlaGlyMetLysProSer
1141  CCCCTTGGAGTGCCTGATGCTGGGATGAAGCCCAGTTAACCAGGCCGGTGTGGGCTGTGT
1201  CGTAGCCAAGGTGGCTGAGCCCTGGCAGGATGACCCTGCGAAGGGGCCCTGGTCCTTCCA
1261  GGCCCCCACCACTAGGACTCTGAGGCTCTTTCTGGGCCAAGTTCCTCTAGTGCCCTCCAC
1321  AGCCGCAGCCTCCCTCTGACCTGCAGGCCAAGAGCAGAGGCAGCGAGTTGTGGAAAGCCT
1381  CTGCTGCCATGGCGTGTCCCTCTCGGAAGGCTGGCTGGGCATGGACGTTCGGGGCATGCT
1441  GGGGCAAGTCCCTGAGTCTCTGTGACCTGCCCCGCCCAGCTGCACCTGCCAGCCTGGCTT
1501  CTGGAGCCCTTGGGTTTTTTGTTTGTTTGTTTGTTTGTTTGTTTGTTTCTCCCCCTGGGC
1561  TCTGCCCAGCTCTGGCTTCCAGAAAACCCCAGCATCCTTTTCTGCAGAGGGGCTTTCTGG
1621  AGAGGAGGGATGCTGCCTGAGTCACCCATGAAGACAGGACAGTGCTTCAGCCTGAGGCTG
1681  AGACTGCGGGATGGTCCTGGGGCTCTGTGCAGGGAGGAGGTGGCAGCCCTGTAGGGAACG
1741  GGGTCCTTCAAGTTAGCTCAGGAGGCTTGGAAAGCATCACCTCAGGCCAGGTGCAGTGGC
1801  TCACGCCTATGATCCCAGCACTTTGGGAGGCTGAGGCGGGTGGATCACCTGAGGTTAGGA
1861  GTTCGAGACCAGCCTGGCCAACATGGTAAAACCCCATCTCTACTAAAAATACAGAAATTA
```

FIGURE 4D

1921 GCCGGGGCGTGGTGGCGGGCACCTATAGTCCCAGCTACTCAGAAGCCTGAGGCTGGGAAAT
1981 CGTTTGAACCCCGGGAAGCGGAGGTTGCAGGGAGCCGAGATCACGCCACTGCACTCCAGCC
2041 TGGGCGACAGAGCGAGAGTCTGTCTCAAAAGAAAAAAAAAAAGCACCGCCTCCAAATGCT
2101 AACTTGTCCTTTTTGTACCATGGTGTGAAAGTCAGATGCCCCAGAGGGCCCAGGCAGGCCAC
2161 CATATTCAGTGCTGTGGCCTGGGCAAGATAACGCACTTCTAACTAGAAATCTGCCCAATTT
2221 TTTAAAAAAGTAAGTACCACTCAGGCCAACAAGCCAACGACAAAGCCAAACTCTGCCAGC
2281 CACATCCAACCCCCCACCTGCCATTTGCACCCTCCGCCTTCACTCCGGTGTGCCTGCAG

```
  1  MAPVAVWAAL AVGLELWAAA HALPAQVAPT PYAPEPGSTC RLREYYDQTA
 51  QMCCSKCSPG QHAKVFCTKT SDTVCDSCED STYTQLWNWV PECLSCGSRC
101  SSDQVETQAC TREQNRICTC RPGWYCALSK QEGCRLCAPL RKCRPGFGVA
151  RPGTETSDVV CKPCAPGTFS NTTSSTDICR PHQICNVVAI PGNASMDAVC
201  TSTSPTRSMA PGAVHLPQPV STRSQHTQPT PEPSTAPSTS FLLPMGPSPP
251  AEGSTGDFAL PVGLIVGVTA LGLLIIGVVN CVIMTQVKKK PLCLQREAKV
301  PHLPADKARG TQGPEQQHLL ITAPSSSSSS LESSASALDR RAPTRNQPQA
351  PGVEASGAGE ARASTGSSDS SPGGHGTQVN VTCIVNVCSS SDHSSQCSSQ
401  ASSTMGDTDS SPSESPKDEQ VPFSKEECAP RSQLETPETL LGSTEEKPLP
451  LGVPDAGMKP S
```

**FIGURE 5**

FIGURE 6A

```
  1 S  D  T  V  C  D  S  C  E  D  S  T  Y  T  Q  L  W  N  W  V
  1 tcggacaccgtgtgtgactcctgtgaggacagcacatacacccagctctggaactgggtt
  1          10         20         30         40         50

 21 P  E  C  L  S  C  G  S  R  C  S  S  D  Q  V  E  T  Q  A  C
 61 cccgagtgcttgagctgtggctcccgctgtagctctgaccaggtggaaactcaagcctgc
 61          70         80         90         100        110

 41 T  R  E  Q  N  R  I  C  T  C  R  P  G  W  Y  C  A  L  S  K
121 actcgggaacagaaccgcatctgcacctgcaggcccggctggtactgcgcgctgagcaag
121          130        140        150        160        170

 61 Q  E  G  C  R  L  C  A  P  L  P  K  C  R  P  G  F  G  V  A
181 caggaggggtgccggctgtgcgccgctgccgaagtgccgcccgggcttcggcgtggcc
181          190        200        210        220        230

 81 R  P  G  T  E  T  S  D  V  V  C  K  P  C  A  P  G  T  F  S
241 agaccaggaactgaaacatcagacgtggtgtgcaagccctgtgccccggggacgttctcc
241          250        260        270        280        290

101 N  T  T  S  S  T  D  I  C  R  P  H  Q  I  C  N  V  V  A  I
301 aacacgacttcatccacggatatttgcaggccccaccagatctgtaacgtggtggccatc
301          310        320        330        340        350
```

**FIGURE 6B**

```
121 P  G  N  A  S  R  D  A  V  C  T  S  T  S  P  T  R  S  M  A
361 cctgggaatgcaagcagggatgcagtctgcacgtccacgtcccccacccggagtatggcc
361          370        380        390        400        410

141 P  G  A  V  H  L  P  Q  P  V  S  T  R  S  Q  H  T  Q  P  S
421 ccaggggcagtacacttaccccagccagtgtccacacgatcccaacacacgcagccaagt
421          430        440        450        460        470

161 P  E  P  S  T  A  P  S  T  S  F  L  L  P  M  G  P  S  P  P
481 ccagaacccagcactgctccaagcacctccttcctgctcccaatgggccccagcccccca
481          490        500        510        520        530

181 A  E  G  S  T  G  D  F  A  L  P  V  G  L  I  V  G  V  T  A
541 gctgaagggagcactggcgacttcgctcttccagttggactgattgtgggtgtgacagcc
541          550        560        570        580        590

201 L  G  L  L  I  I  G  V  V  N  C  V  I  M  T  Q  V  K  K  K
601 ttgggtctactaataataggagtggtgaactgtgtcatcatgacccaggtgaaaaagaag
601          610        620        630        640        650

221 P  L  C  L  Q  R  E  A  K  V  P  H  L  P  A  D  K  A  R  G
661 cccttgtgcctgcagagagaagccaaggtgcctcacttgcctgccgataaggcccggggt
661          670        680        690        700        710
```

FIGURE 6C

```
241 T  Q  G  P  E  Q  Q  H  L  L  I  T  A  P  S  S  S  S  S  S
721 acacagggccccgagcagcagcacctgctgatcacagcgccgagctccagcagcagctcc
721          730       740       750       760       770

261 L  E  S  S  A  S  A  L  D  R  R  A  P  T  R  N  Q  P  Q  A
781 ctggagagctcggccagtgcgttggacagaagggcgcccactcggaaccagccacaggca
781          790       800       810       820       830

281 P  G  V  E  A  S  G  A  G  E  A  R  A  S  T  G  S  S  A  D
841 ccaggcgtggaggccagtggggccggggaggcccgggccagcaccgggagctcagcagat
841          850       860       870       880       890

301 S  S  P  G  G  H  G  T  Q  V  N  V  T  C  I  V  N  V  C  S
901 tcttcccctggtggccatgggacccaggtcaatgtcacctgcatcgtgaacgtctgtagc
901          910       920       930       940       950

321 S  S  D  H  S  S  Q  C  S  S  Q  A  S  S  T  M  G  D  T  D
961 agctctgaccacagctcacagtgctcctcccaagccagctccacaatgggagacacagat
961          970       980       990       1000      1010

341 S  S  P  S  E  S  P  K  D  E  Q  V  P  F  S  K  E  E  C  A
1021 tccagcccctcggagtccccgaaggacgagcaggtccccttctccaaggaggaatgtgcc
1021         1030      1040      1050      1060      1070
```

FIGURE 6D

```
 361 F  R  S  Q  L  E  T  P  E  T  L  L  G  S  T  E  E  K  P  L
1081 tttcggtcacagctggagacgccagagaccctgctggggagcaccgaagagaagcccctg
1081          1090       1100       1110       1120       1130

 381 P  L  G  V  P  D  A  G  M  K  P  S
1141 ccccttggagtgcctgatgctgggatgaagcccagttaaccaggccggtgtgggctgtgt
1141          1150       1160       1170       1180       1190
1201 cgtagccaaggtggctgagccctggcaggatgaccctgcgaaggggccctggtccttcca
1201          1210       1220       1230       1240       1250
1261 ggcccccaccactaggactctgaggctctttctgggccaagttcctctagtgccctccac
1261          1270       1280       1290       1300       1310
1321 agccgcagcctccctctgacctgcaggccaagagcagaggcagcgagttgtggaaagcct
1321          1330       1340       1350       1360       1370
1381 ctgctgccatggcgtgtccctctcggaaggctggctgggcatggacgttcggggcatgct
1381          1390       1400       1410       1420       1430
1441 ggggcaagtccctgagtctctgtgacctgccccgcccagctgcacctgccagcctggctt
1441          1450       1460       1470       1480       1490
1501 ctggagcccttgggtttttgtttgtttgtttgtttgtttgtttctccccctgggc
1501          1510       1520       1530       1540       1550
1561 tctgcccagctctggcttccagaaaaccccagcatcctttctgcagaggggctttctgg
1561          1570       1580       1590       1600       1610
1621 agaggagggatgctgcctgagtcacccatgaagacaggacagtgcttcagcctgaggctg
1621          1630       1640       1650       1660       1670
```

**FIGURE 6E**

```
1681 agactgcgggatggtcctggggctctgtgcagggaggaggtggcagccctgtagggaacg
1681          1690      1700      1710      1720      1730
1741 gggtccttcaagttagctcaggaggcttggaaagcatcacctcaggccaggtgcagtggc
1741          1750      1760      1770      1780      1790
1801 tcacgcctatgatcccagcactttgggaggctgaggcgggtggatcacctgaggttagga
1801          1810      1820      1830      1840      1850
1861 gttcgagaccagcctggccaacatggtaaaaccccatctctactaaaaatacagaaatta
1861          1870      1880      1890      1900      1910
1921 gccgggcgtggtggcgggcacctatagtcccagctactcagaagcctgaggctgggaaat
1921          1930      1940      1950      1960      1970
1981 cgtttgaacccgggaagcggaggttgcagggagccgagatcacgccactgcactccagcc
1981          1990      2000      2010      2020      2030
2041 tgggcgacagagcgagagtctgtctcaaaagaaaaaaaaaaagcaccgcctccaaatgct
2041          2050      2060      2070      2080      2090
2101 aacttgtccttttgtaccatggtgtgaaagtcagatgcccagagggcccaggcaggccac
2101          2110      2120      2130      2140      2150
2161 catattcagtgctgtggcctgggcaagataacgcacttctaactagaaatctgccaattt
2161          2170      2180      2190      2200      2210
2221 tttaaaaaagtaagtaccactcaggccaacaagccaacgacaaagccaaactctgccagc
2221          2230      2240      2250      2260      2270
2281 cacatccaacccccccacctgccatttgcaccctccgccttcactccggtgtgcctgcag
2281          2290      2300      2310      2320      2330
```

# HUMAN TNF RECEPTOR

This is a division of application Ser. No. 08/095,640, filed Jul. 21, 1993; now U.S. Pat. No. 5,610,279, which is a continuation application of Ser. No. 07/580,013, filed Sep. 10, 1990, now abandoned. This application claims priority under 35 U.S.C. §119 to application Ser. Nos. 3319/89, 746/90 and 1347/90, filed on Sep. 12, 1989, Mar. 8, 1990 and Apr. 20, 1990, respectively, all in Switzerland. This application also claims priority under 35 U.S.C. §119 to European Patent Application Number 90116707.2 (now Patent Number EP 0417563), filed Aug. 31, 1990.

## BACKGROUND OF THE INVENTION

Tumor necrosis factor α (TNFα, also cachectin), discovered as a result of its haemorragic-necrotizing activity on certain tumors, and lymphotoxin (TNFβ) are two closely related peptide factors [3] from the class of lymphokines/cytokines which are both referred to hereinafter as TNF [see references 2 and 3]. TNF possesses a broad cellular spectrum of activity. For example, TNF has inhibitory or cytotoxic activity on a series of tumor cell lines [2, 3], stimulates the proliferation of fibroblasts and the phagocytic/cytotoxic activity of myeloic cells [4, 5, 6], induces adhesion molecules in endothelial cells or exerts an inhibitory activity on the endothelium [7, 8, 9, 10], inhibits the synthesis of specific enzymes in adipocytes [11] and induces the expression of histocompatibility antigens [12]. Many of these TNF activities are produced via induction of other factors or by synergistic effects with other factors such as interferons or interleukins [13-16].

TNF is involved in pathological conditions such as shock states in meningococcal sepsis [17], the development of autoimmune glomerulonephritis in mice [18] and cerebral malaria in mice [19] and human beings [41]. The toxic effects of endotoxin appear to be mediated by TNF [20]. Furthermore, TNF can trigger interleukin-1 fever [39]. On the basis of its pleiotropic functional properties, TNF in interaction with other cytokines is involved in additional pathological conditions as a mediator of immune response, inflammation, and other processes.

These biological effects are mediated by TNF via specific receptors. According to present knowledge not only TNFα, but also TNFβ bind to the same receptors [21]. Different cell types differ in their number of TNF receptors [22, 23, 24]. Generally known TNF-binding proteins (TNF-BP) have been detected by covalent bonding to radioactively labelled TNF [24-29], and the following apparent molecular weights of the TNF/TNF-BP complexes obtained have been determined to be: 95/100 kD and 75 kD [24], 95 kD and 75 kD [25], 138 kD, 90 kD, 75 kD and 54 kD [26], 100±5 kD [27], 97 kD and 70 kD [28] and 145 kD [29]. One such TNF/TNF-BP complex was isolated by anti-TNF-antibody immune affinity chromatography and preparative SDS-polyacrylamide gel electrophoreses (SDS-PAGE) [27]. The reductive cleavage of this complex and subsequent SDS-PAGE analysis gave several bands which were not tested for TNF-binding activity. Since the specific conditions which must be used for the cleavage of the complex lead to inactivation of the binding protein [31], the latter has also not been possible. The separation of soluble TNF-BP from human serum or urine by ion exchange chromatography and gel filtration (molecular weight in the region of 50 kD) was described by Olsson et al. [30].

Brockhaus et al. [32] obtained an enriched TNF-BP preparation from membrane extracts of $HL_{60}$ cells by TNFα-ligand affinity chromatography and HPLC which, in turn, was used as an antigen preparation for the production of monoclonal antibodies against TNF-BP. Using such an immobilized antibody (immune affinity chromatography) Loetscher and Brockhaus obtained an enriched preparation of TNF-BP [31] from an extract of human placenta using TNFα-ligand affinity chromatography and HPLC, which gave a strong broad band at 35 kD, a weak band at about 40 kD and a very weak band in the region between 55 kD and 60 kD on SDS-PAGE analysis. Moreover, the gel showed a protein background smear in the region of 33 kD to 40 kD. The significance of these protein bands was, however, not clear due to the heterogenicity of the starting material which was used (placenta tissue; combined material from several placentas). In the state of the art TNF-BP have already been characterized by a N-terminal partial sequence [European Patent Application, Publication No. 308 378], whereby this sequence differs from the N-terminal partial sequence according to formula (IA) in accordance with the invention. Moreover, the TNF-binding proteins described in the state of the art are soluble, i.e. non-membrane bound, TNF-BP and not membrane-bound, i.e. insoluble, TNF-BP isolated from urine.



## SUMMARY OF THE INVENTION

This invention comprises insoluble, homogenous proteins or soluble or insoluble fragments thereof, capable of binding tumor necrosis factor-(TNF).

This invention also comprises TNF-binding proteins containing amino acid sequences of FIG. **1** or FIG. **4**, proteins containing fragments of these sequences, and proteins analagous to the sequences of FIG. **1** or FIG. **4** or to fragments thereof.

This invention further comprises DNA sequences encoding the proteins described above, proteins encoded by these sequences, and antibodies to any of these proteins.

This invention comprises DNA sequences which combine two partial DNA sequences, one sequence encoding soluble fragments of TNF binding proteins and the other partial sequence encoding all domains except the first domain of the constant region of the heavy chain of human immunoglobulin IgG, IgA, IgM, or IgE, and the recombinant proteins encoded by these sequences.

This invention additionally comprises vectors containing the above DNA sequences, and host systems transfected with such vectors.

This invention finally comprises a process for the isolation of an insoluble homogenous protein capable of binding TNF.

## BRIEF DESCRIPTION OF THE FIGURES

FIG. **1**A-**1**D. Nucleotide sequence (SEQ ID NO: 1) and deduced amino acid sequence (SEQ ID NO: 2) for cDNA clone derived from 55 kD TNF-BP. The 19 amino acid transmembrane region is underlined. Hypothetical glycosylation sites are identified by asterisks.

FIG. **2**. Binding analysis of COS cells transfected with plasmid pN123. Panel 2A—binding of transfected cells to $^{125}$I-TNFα. Panel 2B—Scatchard plot of binding data.

FIG. **3**. Sandwich assays of cells transfected with plasmid pK19. Culture supernatants of cells transfected with pK19 were incubated with anti-55 kD TNF-BP antibody followed by $^{125}$I-TNFα. Columns 1, 5, and 8 are controls. Columns 2, 3, 4, 5, and 6 are five parallel transfections.

FIG. **4**A-**4**D. Nucleotide sequence (SEQ ID NO: 28) and deduced amino acid sequence (SEQ ID NO: 29) for cDNA clones derived from 75/65 kD TNF-BP.

FIG. **5**. Deduced amino acid sequence (SEQ ID NO: 27) for a 75/65 kD TNF-BP cDNA clone described in Smith et al., Science 248, 1019-1023, (1990). The leader region is singly underlined, the transmembrane domain is shown boxed, and potential N-linked glycosylation sites are doubly underlined.

FIGS. **6**A-**6**E: Corrected nucleotide sequence (SEQ ID NO: 3) and deduced amino acid sequence (SEQ ID NO: 4) of FIG. **4** after repeated sequencing, showing a threonine coded by "ACC" at position 3 instead of a serine coded by "TCC".

## DETAILED DESCRIPTION OF THE INVENTION

The TNF-binding proteins of the present invention are homogenous, insoluble proteins and soluble or insoluble fragments of such proteins which are capable of binding TNF. These proteins have the ability to bind TNF as measured by standard assays.

The TNF-binding proteins of the present invention include homogenous proteins containing the amino acid sequence depicted in FIG. **1** (SEQ ID NO: 2) or in FIG. **4** (SEQ ID NO: 4), proteins containing fragments of either sequence, and analogues of any such proteins for example proteins containing amino acid sequences analogous to the amino acid sequences of FIG. **1** (SEQ ID NO: 2) or FIG. **4** (SEQ ID NO: 4) or to fragments thereof. An analogue is a protein in which one or more amino acids of the sequences depicted in FIG. **1** (SEQ ID NO: 2) or in FIG. **4** (SEQ ID NO: 4) have had their side-groups chemically modified in a known manner, or those in which one or more amino acids have been replaced or deleted, without thereby eliminating TNF-binding ability. Such analogues may be produced by known methods of peptide chemistry, or by known methods of recombinant DNA technology, such as planned mutagenesis.

The TNF binding activity of the proteins of the present invention may be determined using the assay described in Example 1.

TNF-binding proteins of this invention are obtained as follows:

TNF binding proteins may be isolated from tissues and purified to homogeneity, or isolated from cells which contain membrane-bound TNF binding protein, and purified to homogeneity. One possible method for growing cells and isolating cell extract is described in Example 2, however, other cells types and other growth and isolation methods are well known in the art. Purification of TNF-binding proteins from cell extracts may be performed using the methods described in Examples 4, 5, and 6 in combination with the assay described in Example 1. TNF-binding proteins isolated and purified by these methods were sequenced by well-known methods, as described in Example 7. From these amino acid sequences, DNA probes were produced and used to obtain mRNA encoding TNF binding proteins from which cDNA was made, all by known methods described in Examples 8 and 11. Other well-known methods for producing cDNA are known in the art and may effectively be used. In general, any TNF-binding protein can be isolated from any cell or tissue expressing such proteins using a cDNA probe such as the probe described above, isolating mRNA and transcribing the mRNA into cDNA. Thereafter, the protein can be produced by inserting the cDNA into an expression vector as described in Example 9, such as a virus, plasmid, cosmid, or other vector, inserting the expression vector into a cell, such as the COS cell described in Example 9 or the insect cell described in Example 10, proliferating the resulting cells, and isolating the expressed TNF-binding protein from the medium or from cell extract as described above. Alternatively, TNF-binding proteins may be chemically synthesized using the sequence described above and an amino acid synthesizer, or manual synthesis using chemical conditions well known to form peptide bonds between selected amino acids. Analogues and fragments of TNF-binding proteins may be produced by the above methods. In the case of analogues, the proteins may be chemically modified, or modified by genetic engineering as described above. These fragments and analogues may then be tested for TNF-binding activity using methods such as the assay of Example 1.

Finally, monoclonal antibodies directed against TNF-binding proteins, such as the antibodies described in Example 3, may be produced by known techniques, and used to isolate TNF-binding proteins.

In more detail, the proteins of the present invention are non-soluble proteins, i.e. for example membrane proteins or so-called receptors, and soluble or non-soluble fragments thereof, which bind TNF (TNF-BP), in homogeneous form, as well as their physiologically compatible salts. Preferred proteins are those which according to SDS-PAGE under non-reducing conditions are characterized by apparent molecular weights of about 55 kD, 51 kD, 38 kD, 36 kD and 34 kD or 75 kD and 65 kD, especially those with about 55 kD and 75 kD. Furthermore, there are preferred those proteins which are characterized by containing at least one of the following amino acid partial sequences:

(IA) Leu-Val-Pro-His-Leu-Gly-Asp-Arg-Glu-Lys-Arg-Asp-Ser-Val-Cys-Pro-Gln-Gly-Lys-Tyr-Ile-His-Pro-Gln-X-Asn-Ser-Ile (SEQ ID NO: 5)

(IB) Ser-Thr-Pro-Glu-Lys-Glu-Gly-Glu-Leu-Glu-Gly-Thr-Thr-Thr-Lys (SEQ ID NO: 6)

(IIA) Ser-Gln-Leu-Glu-Thr-Pro-Glu-Thr-Leu-Leu-Gly-Ser-Thr-Glu-Glu-Lys-Pro-Leu (SEQ ID NO: 7)

(IIB) Val-Phe-Cys-Thr (SEQ ID NO: 8)

(IIC) Asn-Gln-Pro-Gln-Ala-Pro-Gly-Val-Glu-Ala-Ser-Gly-Ala-Gly-Glu-Ala (SEQ ID NO: 9)

(IID) Leu-pro-Ala-Gln-Val-Ala-Phe-X-Pro-Tyr-Ala-Pro-Glu-Pro-Gly-Ser-Thr-Cys (SEQ ID NO: 9)

(IIE) Ile-X-Pro-Gly-Phe-Gly-Val-Ala-Tyr-Pro-Ala-Leu-Glu (SEQ ID NO: 11)

(IIF) Leu-Cys-Ala-Pro (SEQ ID NO: 12)

(IIG) Val-Pro-His-Leu-Pro-Ala-Asp (SEQ ID NO: 13)

(IIH) Gly-Ser-Gln-Gly-Pro-Glu-Gln-Gln-X-X-Leu-Ile-X-Ala-Pro (SEQ ID NO: 14)

in which X stands for an amino acid residue which could not be unequivocally determined.

A process for the isolation of the TNF-BP in accordance with the invention is also an object of the present invention. This process comprises carrying out essentially the following purification steps in sequence: production of a cell or tissue extract, immune affinity chromatography and/or single or multiple ligand affinity chromatography, high resolution liquid chromatography (HPLC) and preparative SDS-polyacrylamide gel electrophoresis (SDS-PAGE). The combination of the individual purification steps, which are known from the state of the art, is essential to the success of the process in accordance with the invention, whereby individual steps have been modified and improved having regard to the problem to be solved. Thus, for example, the original combined immune affinity chromatography/TNFα-ligand affinity chromatography step originally used for the enrichment of TNF-BP from human placenta [31] has been altered by using a BSA-Sepharose 4B pre-column. For the application of the cell or membrane extract, this pre-column was connected in series with the immune affinity column followed by the ligand affinity column. After the application of the extract the two aforementioned columns were coupled, each eluted and the TNF-BP-active fractions were purified again via a ligand affinity

column. The use of a detergent-containing solvent mixture for the performance of the reversed-phase HPLC step is essential to the invention.

Further, an industrial process for the production of high cell densities of mammalian cells from which TNF-BP can be isolated is also an object of the present invention. Such a process comprises using a medium, which has been developed for the specific growth requirements of the cell line used, in combination with a perfusion apparatus as described e.g. in detail in Example 2. By means of such a process there can be produced, for example, in the case of HL-60 cells up to more than 20-fold higher cell densities than usual.

In addition thereto, the present invention is also concerned with DNA sequences coding for proteins and soluble or non-soluble fragments thereof, which bind TNF. Thereunder there are to be understood, for example, DNA sequences coding for non-soluble proteins or soluble as well as non-soluble fragments thereof, which bind TNF, such DNA sequences being selected from the following:

(a) DNA sequences as given FIG. **1** or FIG. **4** as well as their complementary strands, or those which include these sequences;

(b) DNA sequences which hybridize with sequences defined under (a) or fragments thereof;

(c) DNA sequences which, because of the degeneracy of the genetic code, do not hybridize with sequences as defined under (a) and (b), but which code for polypeptides having exactly the same amino acid sequence.

That is to say, the present invention embraces not only allelic variants, but also those DNA sequences which result from deletions, substitutions and additions from one or more nucleotides of the sequences given in FIG. **1** or FIG. **4**, whereby in the case of the proteins coded thereby there come into consideration, just as before, TNF-BP. One sequence which results from such a deletion is described, for example, in Smith et al., Science 248, 1019-1023, (1990), which is incorporated by reference herein. FIG. **5** (a reproduction of FIG. **3**B of Smith et al.) shows the deduced amino acid sequence (SEQ ID NO: 27) of the cDNA coding region of a human TNF receptor cDNA clone. The leader region is singly underlined, the transmembrane domain is shown boxed, and potential N-linked glycosylation sites are doubly underlined. The entire nucleotide sequence is available upon request and has been deposited at Genbank under Accession Number M32315.

There are preferred first of all those DNA sequences which code for such a protein having an apparent molecular weight of about 55 kD, whereby the sequence given in FIG. **1** is especially preferred, and sequences which code for non-soluble as well as soluble fragments of such proteins. A DNA sequence which codes, for example, for such a non-soluble protein fragment extends from nucleotide −185 to 1122 of the sequence given in FIG. **1**. DNA sequences which code for soluble protein fragments are, for example, those which extend from nucleotide −185 to 633 or from nucleotide −14 to 633 of the sequence given in FIG. **1**. There are also preferred DNA sequences which code for a protein of about 75/65 kD, whereby those which contain the partial cDNA sequences shown in FIG. **4** are preferred. Especially preferred DNA sequences in this case are the sequences of the open reading frame of nucleotide 2 to 1,177. The peptides IIA, IIC, IIE, IIF, IIG and IIH are coded by the partial cDNA sequence in FIG. **4**, whereby the insignificant deviations in the experimentally determined amino acid sequences are based on the cDNA-derived sequence with highest probability from the limited resolution of the gas phase sequencing. DNA sequences which code for insoluble (deposited on Oct. 17, 2006 with the American Type Culture Collection, 12301 Parklawn Drive, Rockville, Md. 20852, under Accession No. PTA 7942) as well as soluble fractions of TNF-binding proteins having an apparent molecular weight of 65 kD/75 kD are also preferred. DNA sequences for such soluble fragments can be determined on the basis of the amino acid sequences derived from the nucleic acid sequences coding for such non-soluble TNF-BP.

The invention is also concerned with DNA sequences which comprise a combination of two partial DNA sequences, with one of the partial sequences coding for those soluble fragments of non-soluble proteins which bind TNF (see above) and the other partial sequence coding for all domains other than the first domain of the constant region of the heavy chain of human immunoglobulins such as IgG, IgA, IgM or IgE, in particular $IgG_1$ or $IgG_3$ subtypes.

The present invention is also concerned with the recombinant proteins coded by any of DNA sequences described above. Of course, there are thereby also included such proteins in whose amino acid sequences amino acids have been exchanged, for example by planned mutagenesis, so that the activity of the TNF-BP or fragments thereof, namely the binding of TNF or the interaction with other membrane components participating in the signal transfer, have been altered or maintained in a desirable manner. Amino acid exchanges in proteins and peptides which do not generally alter the activity of such molecules are known in the state of the art and are described, for example, by H. Neurath and R. L. Hill in "The Proteins" (Academic Press, New York, 1979, see especially FIG. **6**, page 14). The most commonly occurring exchanges are: Ala/Ser, Val/Ile, Asp/Glu, Thr/Ser, Ala/Gly, Ala/Thr, Ser/Asn, Ala/Val, Ser/Gly, Tyr/Phe, Ala/Pro, Lys/Arg, Asp/Asn, Leu/Ile, Leu/Val, Ala/Glu, Asp/Gly as well as these in reverse. The present invention is also concerned with vectors which contain any of the DNA sequences described above in accordance with the invention and which are suitable for the transformation of suitable pro- and eukaryotic host systems, whereby there are preferred those vectors whose use leads to the expression of the proteins which are coded by any of the DNA sequences described above in accordance with the invention. Finally, the present invention is also concerned with pro- and eukaryotic host systems transformed with such vectors, as well as a process for the production of recombinant compounds in accordance with the invention by cultivating such host systems and subsequently isolating these compounds from the host systems themselves or their culture supernatants.

An object of the present invention are also pharmaceutical preparations which contain at least one of these TNF-BPs or fragments thereof, if desired in combination with other pharmaceutically active substances and/or non-toxic, inert, therapeutically compatible carrier materials.

Finally, the present invention is concerned with the use of such a TNF-BP on the one hand for the production of pharmaceutical preparations and on the other hand for the treatment of illnesses, preferably those in which TNF is involved in their course.

Starting materials for the TNF-BP in accordance with the invention are quite generally cells which contain such TNF-BP in membrane-bound form and which are generally accessible without restrictions to a person skilled in the art, such as, for example, HL60 [ATCC No. CCL 240], U 937 [ATCC No. CRL 1593], SW 480 [ATCC No. CCL 228] and HEp2 cells [ATCC No. CCL 23]. These cells can be cultivated according to known methods of the state of the art [40] or, in order to produce high cell densities, according to the procedure already described generally and described in detail in

Example 2 for HL60 cells. TNF-BP can then be extracted from the cells, which are centrifuged-off from the medium and washed, according to known methods of the state of the art using suitable detergents, for example Triton X-114, 1-0-n-octyl-β-D-glucopyranoside (octylglucoside) or 3-[(3-cholylamido-propyl)-dimethylammonio]-1-propane sulphonate (CHAPS), especially using Triton X-100. For the detection of such TNF-BP there can be used the usually used detection methods for TNF-BP, for example a polyethylene glycol-induced precipitation of the $^{125}$I-TNF/TNF-BP complex [27], especially filter-binding tests with radioactively labelled TNF according to Example 1. In order to produce the TNF-BP in accordance with the invention, the general methods of the state of the art used for the purification of proteins, especially of membrane proteins, such as, for example, ion exchange chromatography, gel filtration, affinity chromatography, HPLC and SDS-PAGE can be used. Especially preferred methods for the production of TNF-BP in accordance with the invention are affinity chromatography, especially with TNF-α as the ligand bound to the solid phase, and immune affinity chromatography, HPLC and SDS-PAGE. The elution of TNF-BP bands which are separated using SDS-PAGE can be effected according to known methods of protein chemistry, for example using electroelution according to Hunkapiller et al. [34], whereby according to present knowledge the electro-dialysis times given there generally have to be doubled. Thereafter, traces of SDS which still remain can then be removed in accordance with Bosserhoff et al. [50].

The thus-purified TNF-BP can be characterized by methods of peptide chemistry which are known in the state of the art, such as, for example, N-terminal amino acid sequencing or enzymatic as well as chemical peptide cleavage. Fragments obtained by enzymatic or chemical cleavage can be separated according to usual methods such as, for example, HPLC and can themselves be subjected to further N-terminal sequencing. Such fragments which themselves bind TNF can be identified using the aforementioned detection methods for TNF-BP and are likewise objects of the present invention.

Starting from the thus-obtained amino acid sequence information or the DNA and amino acid sequences given in FIG. **1** as well as in FIG. **4** there can be produced, taking into consideration the degeneracy of the genetic code, according to methods known in the state of the art suitable oligonucleotides [51]. By means of these, again according to known methods of molecular biology [42, 43], cDNA or genomic DNA banks can be searched for clones which contain nucleic acid sequences coding for TNF-BP. Moreover, using the polymerase chain reaction (PCR) [49] cDNA fragments can be cloned by completely degenerating the amino acid sequence of two spaced apart relatively short segments while taking into consideration the genetic code and introducing into their complementarity suitable oligo-nucleotides as a "primer", whereby the fragment lying between these two sequences can be amplified and identified. The determination of the nucleotide sequence of a such a fragment permits an independent determination of the amino acid sequence of the protein fragment for which it codes. The cDNA fragments obtainable by PCR can also, as already described for the oligonucleotides themselves, be used according to known methods to search for clones containing nucleic acid sequences coding for TNF-BP from cDNA or genomic DNA banks. Such nucleic acid sequences can then be sequenced according to known methods [42]. On the basis of the thus-determined sequences and of the already known sequences for certain receptors, those partial sequences which code for soluble TNF-BP fragments can be determined and cut out from the complete sequence using known methods [42].

The complete sequence or such partial sequences can then be integrated using known methods into vectors described in the state of the art for their multiplication and expression in prokaryotes [42]. Suitable prokaryotic host organisms are, for example, gram-negative and gram-positive bacteria such as, for example, *E. coli* strains such as *E. coli* HB101 [ATCC No. 33 694] or *E. coli* W3110 [ATCC No. 27 325] or *B. subtilis* strains.

Furthermore, nucleic acid sequences in accordance with the invention which code for TNF-BP as well as for TNF-BP fragments can be integrated using known methods into suitable vectors for reproduction and expression in eukaryotic host cells, such as, for example, yeast, insect cells and mammalian cells. Expression of such sequences is preferably effected in mammalian and insect cells.

A typical expression vector for mammalian cells contains an efficient promoter element in order to produce a good transcription rate, the DNA sequence to be expressed and signals for an efficient termination and polyadenylation of the transcript. Additional elements which can be used are "enhancers" which lead to again intensified transcription and sequences which e.g. can bring about a longer half life of the mRNA. For the expression of nucleic acid sequences in which the endogenous sequence fragment coding for a signal peptide is missing, there can be used vectors which contain such suitable sequences which code for signal peptides of other known proteins. See, for example, the vector pLJ268 described by Cullen, B. R. in Cell 46, 973-982 (1986) as well as Sharma, S. et al. in "Current Communications in Molecular Biology", edt. by Gething, M. J., Cold Spring Harbor Lab. (1985), pages 73-78.

Most of these vectors which are used for a transient expression of a particular DNA sequence in mammalian cells contain the replication source of the SV40 virus. In cells which express the T-antigen of the virus (e.g. COS cells), these vectors are reproduced abundantly. A transient expression is, however, not limited to COS cells. In principle any transfectable mammalian cell line can be used for this purpose. Signals which can bring about a strong transcription are e.g. the early and late promoters of SV40, the promoter and enhancer of the "major immediate-early" gene of HCMV (human cytomegalovirus), the LTR's ("long terminal repeats") of retroviruses such as, for example, RSV, HIV and MMTV. There can, however, also be used signals of cellular genes such as e.g. the promoters of the actin and collagenase genes.

Alternatively, however, stable cell lines which have the specific DNA sequence integrated into the genome (chromosome) can also be obtained. For this, the DNA sequence is cotransfected together with a selectable marker, e.g. neomycin, hygromycin, dihydrofolate reductase (dhfr) or hypoxanthin guanine phosphoribosyl transferase (hgpt). The DNA sequence stably incorporated in the chromosome can also be reproduced abundantly. A suitable selection marker for this is, for example, dihydrofolate reductase (dhfr). Mammalian cells (e.g. CHO cells), which contain no intact dhfr gene, are thereby incubated with increasing amounts of methotrexate after transinfection has been effected. In this manner cell lines which contain more than a thousand copies of the desired DNA sequence can be obtained.

Mammalian cells which can be used for the expression are e.g. cells of the human cell lines Hela [ATCC CCL2] and 293 [ATCC CRL 1573] as well as 3T3 [ATCC CCL 163] and L cells, e.g. [ATCC CCL 149], (CHO) cells [ATCC CCL 61],

BHK [ATCC CCL 10] cells as well as the CV 1 [ATCC CCL 70] and the COS cell lines [ATCC CRL 1650, CRL 1651].

Suitable expression vectors include, for example, vectors such as pBC12MI [ATCC 67 109], pSV2dhfr [ATCC 37 146], pSVL [Pharmacia, Uppsala, Sweden], pRSVcat [ATCC 37 152] and pMSG [Pharmacia, Uppsala, Sweden]. The vectors "pK19" and "pN123" used in Example 9 are especially preferred vectors. These can be isolated according to known methods from *E. coli* strains HB101(pK19) and HB101 (pN123) transformed with them [42]. These *E. coli* strains have been deposited on the 26 Jan. 1990 at the Deutschen Sammlung von Mikroorganismen und Zellkulturen GmbH (DSM) in Braunschweig, FRG, under DSM 5761 for HB101 (pK19) and DMS 5764 for HB101(pN123). For the expression of proteins which consist of a soluble fragment of non-soluble TNF-BP and an immunoglobulin fragment, i.e. all domains except the first of the constant region of the heavy chain, there are especially suitable pSV2-derived vectors as described, for example, by German, C. in "DNA Cloning" [Vol. II., edt. by Glover, D. M., IRL Press, Oxford, 1985]. The vectors pCD4-Hμ (DSM 5315), pDC4-Hγ1 (DSM 5314) and pCD4-Hγ3 (DSM 5523) which have been deposited at the Deutschen Sammlung von Mikroorganismen und Zellkulturen GmbH (DSM) in Braunschweig, FRG, and which are described in detail in European Patent Application No. 90107393.2 are especially preferred vectors. This European Patent Specification and the equivalent Applications referred to in Example 11 also contain data with respect to the further use of these vectors for the expression of chimeric proteins (see also Example 11) and for the construction of vectors for the expression of such chimeric proteins with other immunoglobulin fragments.

The manner in which these cells are transfected depends on the chosen expression system and vector system. An overview of these methods is to be found e.g. in Pollard et al., "DNA Transformation of Mammalian Cells" in "Methods in Molecular Biology" [Nucleic Acids Vol. 2, 1984, Walker, J. M., ed, Humana, Clifton, N. J.]. Further methods are to be found in Chen and Okayama ["High-Efficiency Transformation of Mammalian Cells by Plasmid DNA", Molecular and Cell Biology 7, 2745-2752, 1987] and in Feigner [Feigner et al., "Lipofectin: A highly efficient, lipid-mediated DNA-transfection procedure", Proc. Nat. Acad. Sci. USA 84, 7413-7417, 1987].

The baculovirus expression system, which has already been used successfully for the expression of a series of proteins (for an overview see Luckow and Summers, Bio/Technology 47-55, 1988), can be used for the expression in insect cells. Recombinant proteins can be produced in authentic form or as fusion proteins. The thus-produced proteins can also be modified such as, for example, glycosylated (Smith et al., Proc. Nat. Acad. Sci. USA 82, 8404-8408, 1987). For the production of a recombinant baculovirus which expresses the desired protein there is used a so-called "transfer vector". Under this there is to be understood a plasmid which contains the heterologous DNA sequence under the control of a strong promoter, e.g. that of the polyhedron gene, whereby this is surrounded on both sides by viral sequences. The vectors "pN113", "pN119" and "pN124" used in Example 10 are especially preferred vectors. These can be isolated according to known methods from *E. coli* strains HB101(pN113), HB101(pN119) and HB101(pN124) transformed with them. These *E. coli* strains have been deposited on the 26 Jan. 1990 at the Deutschen Sammlung von Mikroorganismen and Zellkulturen GmbH (DSM) in Braunschweig, FRG, under DSM 5762 for HB101(pN113), DSM 5763 for HB101 (pN119) and DSM 5765 for HB101(pN124). The transfer vector is then transfected into the insect cells together with DNA of the wild type baculovirus. The recombinant viruses which result in the cells by homologous recombination can then be identified and isolated according to known methods. An overview of the baculovirus expression system and the methods used therein is to be found in Luckow and Summers [52].

Expressed TNF-BP as well as its non-soluble or soluble fractions can then be purified from the cell mass or the culture supernatants according to methods of protein chemistry which are known in the state of the art, such as, for example, the procedure already described on pages 5-6.

The TNF-BP obtained in accordance with the invention can also be used as antigens to produce polyclonal and monoclonal antibodies according to known techniques [44, 45] or according to the procedure described in Example 3. Such antibodies, especially monoclonal antibodies against the 75 kD TNF-BP species, are also an object of the present invention. Those antibodies which are directed against the 75 kD TNF-BP can be used for the isolation of TNF-BP by modifications of the purification procedure described in detail in Examples 4-6 which are familiar to a person skilled in the art.

On the basis of the high binding affinity of TNF-BP in accordance with the invention for TNF ($K_d$ value in the order of $10^{-9}$-$10^{-10}$M), these or fragments thereof can be used as diagnostics for the detection of TNF in serum or other body fluids according to methods known in the state of the art, for example in solid phase binding tests or in combination with anti-TNF-BP antibodies in so-called "sandwich" tests.

Moreover, TNF-BP in accordance with the invention can be used on the one hand for the purification of TNF and on the other hand for the detection of TNF agonists and TNF antagonists according to procedures which are known in the state of the art.

The TNF-BP in accordance with the invention as well as their physiologically compatible salts, which can be manufactured according to methods which are known in the state of the art, can also be used for the production of pharmaceutical preparations, primarily those for the treatment of illnesses in which TNF is involved in their course. For this purpose, one or more of the said compounds, where desired or required in combination with other pharmaceutically active substances, can be processed in a known manner with the usually used solid or liquid carrier materials. The dosage of such preparations can be effected having regard to the usual criteria in analogy to already used preparations of similar activity and structure.

Since the invention has been described hereinbefore in general terms, the following Examples are intended to illustrate details of the invention, but they are not intended to limit its scope in any manner.

## Example 1

### Detection of TNF-Binding Proteins

The TNF-BP were detected in a filter test with human radioiodinated $^{125}$I-TNF. TNF (46, 47) was radioactively labelled with Na$^{125}$ I (IMS40, Amersham, Amersham, England) and iodo gene (#28600, Pierce Eurochemie, Oud-Beijerland, Netherlands) according to Fraker and Speck [48]. For the detection of the TNF-BP, isolated membranes of the cells or their solubilized, enriched and purified fractions were applied to moist nitrocellulose filter (0.45μ, BioRad, Richmond, Calif., USA). The filters were then blocked in buffer solution with 1% skimmed milk powder and subsequently incubated with $5 \cdot 10^5$ cpm/ml of $^{125}$I-TNFα ($0.3\text{-}1.0 \cdot 10^8$ cpm/

μg) in two batches with and without the addition of 5 μg/ml of non-labelled TNFα, washed and dried in the air. The bound radioactivity was detected semiquantitatively by autoradiography or counted in a gamma-counter. The specific $^{125}$I-TNF-α binding was determined after correction for unspecific binding in the presence of unlabelled TNF-α in excess. The specific TNF-binding in the filter test was measured at various TNF concentrations and analyzed according to Scatchard, whereby a $K_d$ value of $\cdot 10^{-9}$-$10^{-10}$M was determined.

## Example 2

### Cell Extracts of HL-60 Cells

HL60 cells [ATCC No. CCL 240] were cultivated on an experimental laboratory scale in a RPMI 1640 medium [GIBCO catalogue No. 074-01800], which contained 2 g/l $NaHCO_3$ and 5% foetal calf serum, in a 5% $CO_2$ atmosphere and subsequently centrifuged.

The following procedure was used to produce high cell densities on an industrial scale. The cultivation was carried out in a 75 l Airlift fermenter (Fa. Chemap, Switzerland) with a working volume of 58 l. For this there was used the cassette membrane system "PROSTAK" (Millipore, Switzerland) with a membrane surface of 0.32 $m^2$ (1 cassette) integrated into the external circulation circuit. The culture medium (see Table 1) was pumped around with a Watson-Marlow pump, Type 603U, with 5 l/min. After a steam sterilization of the installation, whereby the "PROSTAK" system was sterilized separately in autoclaves, the fermentation was started with growing HL-60 cells from a 20 l Airlift fermenter (Chemap). The cell cultivation in the inoculation fermenter was effected in a conventional batch process in the medium according to Table 1 and an initial cell titre of $2\times10^5$ cells/ml. After 4 days the HL60 batch was transferred with a titre of $4.9\times10^6$ cells/ml into the 75 l fermenter. The pH value was held at 7.1 and the $pO_2$ value was held at 25% saturation, whereby the oxygen introduction was effected through a microporous frit. After initial batch fermentation, on the 2nd day the perfusion at a cell titre of $4\times10^6$ cells/ml was started with 30 l of medium exchange per day. On the filtrate side of the medium the conditioned medium was removed and replaced by the addition of fresh medium. The added medium was fortified as follows: Primatone from 0.25% to 0.35%, glutamine from 5 mM to 6 mM and glucose from 4 g/l to 6 g/l. The perfusion rate was then increased on the 3rd and 4th day to 72 l of medium/day and on the 5th day to 100 l of medium/day. The fermentation had finished after 120 hours of continuous cultivation. Exponential cell growth up to $40\times10^6$ cells/ml took place under the given fermentation conditions. The duplication time of the cell population was 20-22 hours to $10\times10^6$ cells/ml and then increased to 30-36 hours with increasing cell density. The proportion of living cells lay at 90-95% during the entire fermentation period. The HL-60 batch was then cooled down in the fermenter to about 12° C. and the cells were harvested by centrifugation (Beckman centrifuge [Model J-6B, Rotor JS], 3000 rpm, 10 min., 4° C.).

TABLE 1

HL-60 medium

| Components | Concentrations mg/l |
|---|---|
| $CaCl_2$ (anhydrous) | 112.644 |
| $Ca(NO_3)_2 \cdot 4H_2O$ | 20 |
| $CuSO_4 \cdot 5H_2O$ | $0.498\cdot10^{-3}$ |
| $Fe(NO_3)_3 \cdot 9H_2O$ | 0.02 |
| $FeSO_4 \cdot 7H_2O$ | 0.1668 |
| KCl | 336.72 |
| $KNO_3$ | 0.0309 |
| $MgCl_2$ (anhydrous) | 11.444 |
| $MgSO_4$ (anhydrous) | 68.37 |
| NaCl | 5801.8 |
| $Na_2HPO_4$ (anhydrous) | 188.408 |
| $NaH_2PO_4 \cdot H_2O$ | 75 |
| $Na_2SeO_3 \cdot 5H_2O$ | $9.6\cdot10^{-3}$ |
| $ZnSO_4 \cdot 7H_2O$ | 0.1726 |
| D-Glucose | 4000 |
| Glutathion (red.) | 0.2 |
| Hepes buffer | 2383.2 |
| Hypoxanthin | 0.954 |
| Linoleic acid | 0.0168 |
| Lipoic acid | 0.042 |
| Phenol Red | 10.24 |
| Putrescine 2HCl | 0.0322 |
| Na pyruvate | 88 |
| Thymidine | 0.146 |
| Biotin | 0.04666 |
| D-Ca pantothenate | 2.546 |
| Choline chloride | 5.792 |
| Folic acid | 2.86 |
| i-Inositol | 11.32 |
| Niacinamide | 2.6 |
| Nicotinamide | 0.0074 |
| para-Aminobenzoic acid | 0.2 |
| Pyridoxal HCl | 2.4124 |
| Pyridoxin HCl | 0.2 |
| Riboflavin | 0.2876 |
| Thiamin HCl | 2.668 |
| Vitamin $B_{12}$ | 0.2782 |
| L-Alanine | 11.78 |
| L-Aspartic acid | 10 |
| L-Asparagine $H_2O$ | 14.362 |
| L-Arginine | 40 |
| L-Arginine HCl | 92.6 |
| L-Aspartate | 33.32 |
| L-Cystine 2HCl | 62.04 |
| L-Cysteine HCl$\cdot H_2O$ | 7.024 |
| L-Glutamic acid | 36.94 |
| L-Glutamine | 730 |
| L-Glycine | 21.5 |
| L-Histidine | 3 |
| L-Histidine HCl$\cdot H_2O$ | 27.392 |
| L-Hydroxypyroline | 4 |
| L-Isoleucine | 73.788 |
| L-Leucine | 75.62 |
| L-Lysine HCl | 102.9 |
| L-Methionine | 21.896 |
| L-Phenylalanine | 43.592 |
| L-Proline | 26.9 |
| L-Serine | 31.3 |
| L-Threonine | 53 |
| L-Tryptophan | 11.008 |
| L-Tyrosine•2Na | 69.76 |
| L-Valine | 62.74 |
| Penicillin/streptomycin | 100 U/ml |
| Insulin (human) | 5 μg/ml |
| Tranferrin (human) | 15 pg/ml |
| Bovine serum albumin | 67 pg/ml |
| Primatone RL (Sheffield Products, Norwich NY, USA) | 0.25% |
| Pluronic F68 (Serva, Heidelberg, FRG) | 0.01% |
| Foetal calf serum | 0.3-3% |

The centrifugate was washed with isotonic phosphate buffer (PBS; 0.2 g/l KCl, 0.2 g/l $KH_2PO_4$, 8.0 g/l NaCl, 2.16 g/l $Na_2HPO_4.7H_2O$), which had been treated with 5% dim-

ethylformamide, 10 mM benzamidine, 100 U/ml aprotinin, 10 μM leupeptin, 1 μM pepstatin, 1 mM o-phenanthroline, 5 mM iodoacetamide, 1 mM phenyl-methylsulphonyl fluoride (referred to hereinafter as PBS-M). The washed cells were extracted at a density of $2.5 \cdot 10^8$ cells/ml in PBS-M with Triton X-100 (final concentration 1.0%). The cell extract was clarified by centrifugation (15,000×g, 1 hour; 100,000×g, 1 hour).

## Example 3

### Production of Monoclonal (TNF-BP) Antibodies

A centrifugation supernatant from the cultivation of HL60 cells on an experimental laboratory scale, obtained according to Example 2, was diluted with PBS in the ratio 1:10. The diluted supernatant was applied at 4° C. (flow rate: 0.2 ml/min.) to a column which contained 2 ml of Affigel 10 (Bio Rad Catalogue No. 153-6099) to which had been coupled 20 mg of recombinant human TNF-α [Pennica, D. et al. (1984) Nature 312, 724; Shirai, T. et al. (1985) Nature 313, 803; Wang, A. M. et al. (1985) Science 228, 149] according to the recommendations of the manufacturer. The column was washed at 4° C. and a throughflow rate of 1 ml/min firstly with 20 ml of PBS which contained 0.1% Triton X 114 and thereafter with 20 ml of PBS. Thus-enriched TNF-BP was eluted at 22° C. and a flow rate of 2 ml/min with 4 ml of 100 mM glycine, pH 2.8, 0.1% decylmaltoside. The eluate was concentrated to 10 μl in a Centricon 30 unit [Amicon].

10 μl of this eluate were mixed with 20 μl of complete Freund's adjuvant to give an emulsion. 10 μl of the emulsion were injected according to the procedure described by Holmdahl, R. et al. [(1985), J. Immunol. Methods 83, 379] on each of days 0, 7 and 12 into a hind paw of a narcotized Balb/c mouse.

The immunized mice were sacrificed on day 14, the popliteal lymph nodes were removed, minced and suspended by repeated pipetting in Iscove's medium (IMEM, GIBCO Catalogue No. 074-2200) which contained 2 g/l $NaHCO_3$. According to a modified procedure of De St. Groth and Scheidegger [J. Immunol. Methods (1980), 35, 1] $5 \times 10^7$ cells of the lymph nodes were fused with $5 \times 10^7$ PAI mouse myeloma cells (J. W. Stocker et al. Research Disclosure, 217, May 1982, 155-157) which were in logarithmic growth. The cells were mixed, collected by centrifugation and resuspended in 2 ml of 50% (v/v) polyethylene glycol in IMEM at room temperature by slight shaking and diluted by the slow addition of 10 ml of IMEM during careful shaking for 10 minutes. The cells were collected by centrifugation and resuspended in 200 ml of complete medium [IMEM+20% foetal calf serum, glutamine (2.0 mM), 2-mercaptoethanol (100 μM), 100 μM hypoxanthine, 0.4 μM aminopterine and 16 μM thymidine (HAT)]. The suspension was distributed on 10 tissue culture dishes each containing 96 wells and incubated at 37° C. for 11 days without changing the medium in an atmosphere of 5% $CO_2$ and a relative humidity of 98%.

The antibodies are distinguished by their inhibitory action on the binding of TNF to HL60 cells or by their binding to antigens in the filter test according to Example 1. The following procedure was used to detect the biological activity of anti(TNF-BP) antibodies: $5 \times 10^6$ HL60 or U937 cells were incubated in complete RPMI 1640 medium together with affinity-purified monoclonal anti-(TNF-BP) antibodies or control antibodies (i.e. those which are not directed against TNF-BP) in a concentration range of 1 ng/ml to 10 μg/ml. After incubation at 37° C. for one hour the cells were collected by centrifugation and washed with 4.5 ml of PBS at 0° C. They were resuspended in 1 ml of complete RPMI 1640 medium (Example 2) which additionally contained 0.1% sodium azide and $^{125}$I-TNFα ($10^6$ cpm/ml) with or without the addition of unlabelled TNFα (see above). The specific radioactivity of the $^{125}$I-TNFα amounted to 700 Ci/mmol. The cells were incubated at 4° C. for 2 hours, collected and washed 4 times at 0° C. with 4.5 ml of PBS which contained 1% BSA and 0.001% Triton X 100 (Fluke). The radioactivity bound to the cells was measured in a γ-scintillation counter. The cell-bound radioactivity of cells which had not been treated with anti-(TNF-BP) antibodies was determined in a comparative experiment (approximately 10 000 cpm/$5 \times 10^6$ cells).

## Example 4

### Affinity Chromatography

For the further purification, a monoclonal anti-(55 kD TNF-BP) antibody (2.8 mg/ml gel), obtained according to Example 3, TNFα (3.9 mg/ml gel) and bovine serum albumin (BSA, 8.5 mg/ml gel) were each covalently coupled to CNBr-activated Sepharose 4B (Pharmacia, Uppsala, Sweden) according to the directions of the manufacturer. The cell extract obtained according to Example 2 was passed through the thus-prepared columns which were connected in series in the following sequence: BSA-Sepharose pre-column, immune affinity column [anti-(55 kD-TNF-BP) antibody], TNFα-ligand affinity column. After completion of the application the two last-mentioned columns were separated and washed individually with in each case 100 ml of the following buffer solutions: (1) PBS, 1.0% Triton X-100, 10 mM benzamidine, 100 U/ml aprotinin; (2) PBS, 0.1% Triton X-100, 0.5M NaCl, 10 mM ATP, 10 mM benzamidine, 100 U/ml aprotinin; and (3) PBS, 0.1% Triton X-100, 10 mM benzamidine, 100 U/ml aprotinin. Not only the immune affinity column, but also the TNFα-ligand affinity column were then each eluted with 100 mM glycine pH 2.5, 100 mM NaCl, 0.2% decylmaltoside, 10 mM benzamidine, 100 U/ml aprotinin. The fractions of each column which were active in the filter test according to Example 1 were thereafter combined and neutralized with 1M Tris pH 8.0.

The thus-combined TNF-BP active fractions of the immune affinity chromatography on the one hand and of the TNFα-ligand affinity chromatography on the other hand were, for further purification, again applied to in each case one small TNFα-ligand affinity column. Thereafter, these two columns were washed with in each case 40 ml of (1) PBS, 1.0% Triton X-100, 10 mM benzamidine, 100 U/ml aprotinin, (2) PBS, 0.1% Triton X-100, 0.5M NaCl, 10 mM ATP, 10 mM benzamidine, 100 U/ml aprotinin, (3) PBS, 0.1% Triton X-100, (4) 50 mM Tris pH 7.5, 150 mM NaCl, 1.0% NP-40, 1.0% desoxycholate, 0.1% SDS, (5) PBS, 0.2% decylmaltoside. Subsequently, the columns were eluted with 100 mM glycine pH 2.5, 100 mM NaCl, 0.2% decylmaltoside. Fractions of 0.5 ml from each column were collected and the fractions from each column which were active according to the filter test (Example 1) were combined and concentrated in a Centricon unit (Amicon, molecular weight exclusion 10,000).

## Example 5

### Separation by Means of HPLC

The active fractions obtained according to Example 4 were each applied according to their different source (immune or

ligand affinity chromatography) to C1/C8 reversed phase HPLC-columns (ProRPC, Pharmacia, 5×20 mm) which had been equilibrated with 0.1% trifluoroacetic acid, 0.1% octyl-glucoside. The columns were then eluted with a linear aceto-nitrile gradient (0-80%) in the same buffer at a flow of 0.5 ml/min. Fractions of 1.0 ml were collected from each column and the active fractions from each column were combined (detection according to Example 1).

## Example 6

### Separation by Means of SDS-PAGE

The fractions which were obtained according to Example 5 and which were active according to the filter test (Example 1) were further separated by SDS-PAGE according to [34]. For this purpose, the samples were heated to 95° C. for 3 minutes in SDS sample buffer and subsequently separated electro-phoretically on a 12% acrylamide separation gel with a 5% collection gel. The following standard proteins were used as a reference for the determination of the apparent molecular weights on the SDS-PAGE gel: phosphorylase B (97.4 kD), BSA (66.2 kD), ovalbumin (42.7 kD), carboanhydrase (31.0 kD), soya trypsin inhibitor (21.5 kD) and lysozyme (14.4 kD).

Under the mentioned conditions there were obtained for samples which has been obtained according to Example 4 by TNF-α-ligand affinity chromatography of immune affinity chromatography eluates and which had been further separated by HPLC according to Example 5 two bands of 55 kD and 51 kD as well as three weaker bands of 38 kD, 36 kD and 34 kD. These bands were transferred electrophoretically during 1 hour at 100 V in 25 mM Tris, 192 mM glycine, 20% methanol on to a PVDF membrane (Immobilon, Millipore, Bedford, Mass. USA) in a Mini Trans Blot System (BioRad, Richmond, Calif., USA). Thereafter, the PVDF membrane was either protein-stained with 0.15% Serva-Blue (Serva, Heidelberg, FRG) in methanol/water/glacial acetic acid (50/40/10 parts by volume) or blocked with skimmed milk powder and subsequently incubated with $^{125}$I-TNFα according to the filter test conditions described in Example 1 in order to detect bands having TNF-BP activity. This showed that all bands produced in the protein staining bonded TNFα specifically. In the Western blot according to Towbin et al. [38] all of these bands also bonded the monoclonal anti-55 kD-TNF-BP antibody produced according to Example 3. In this case, a procedure according to that described in Example 1 with Na$^{125}$I radioactively-labelled, affinity-purified (mouse immunoglobulin-Sepharose-4B affinity column) rabbit-anti-mouse-immunoglobulin antibody was used for the autoradiographic detection of this antibody.

Samples which had been obtained according to Example 4 by two-fold TNF-α-ligand affinity chromatography of the throughput of the immune affinity chromatography and which had been further separated by HPLC according to Example 5 showed under the above-specified SDS-PAGE and blot transfer conditions two additional bands of 75 kD and 65 kD, both of which bonded TNF specifically in the filter test (Example 1). In the Western blot according to Towbin et al. (see above) the proteins of these two bands did not react with the anti-(55 kD TNF-BP) antibody produced according to Example 3. They reacted, however, with a monoclonal antibody which had been produced starting from the 75 kD band (anti-75 kD TNF-BP antibody) according to Example 3.

## Example 7

### Amino Acid Sequence Analysis

For the amino acid sequence analysis, the fractions which had been obtained according to Example 5 and which were active according to the filter test (Example 1) were separated using the SDS-PAGE conditions described in Example 6, but now reducing (SDS sample buffer with 125 mM dithiothreitol). The same bands as in Example 6 were found, but because of the reducing conditions of the SDS-PAGE in comparison to Example 6 all showed an about 1-2 kD higher molecular weight. These bands were then transferred according to Example 6 on to PVDF membranes and stained with 0.15% Serva-Blue in methanol/water/glacial acetic acid (50/400/10 parts by volume) for 1 minute, decolorized with methanol/water/glacial acetic acid (45/48/7 parts by volume), rinsed with water, dried in air and thereafter cut out. The conditions given by Hunkapiller [34] were adhered to in all steps in order to avoid N-terminal blocking. Initially, the purified TNF-BP were used unaltered for the amino acid sequencing. In order to obtain additional sequence information, the TNF-BP after reduction and S-carboxymethylation [Jones, B. N. (1986) in "Methods of Protein Micro-characterisation", J. E. Shively, ed., Humana Press, Clifton N. J., 124-125] were cleaved with cyanogen bromide (Tarr, G. E. in "Methods of Protein Micro-characterisation", 165-166, loc. cit.), trypsin and/or proteinase K and the peptides were separated by HPLC according to known methods of protein chemistry. Thus-prepared samples were then sequenced in an automatic gas phase microsequencing apparatus (Applied Biosystems Model 470A, ABI, Foster City, Calif., USA) with an on-line automatic HPLC PTH amino acid analyzer (Applied Biosystems Model 120, ABI see above) connected to the outlet, whereby the following amino acid sequences were determined:

1. For the 55 kD band (according to non-reducing SDS-PAGE):

Leu-Val-Pro-His-Leu-Gly-Asp-Arg-Glu-Lys-Arg-Asp-Ser-Val-Cys-Pro-Gln-Gly-Lys-Tyr-Ile-His-Pro-Gln-X-Asn-Ser-Ile (SEQ ID NO: 5),

and

Ser-Thr-Pro-Glu-Lys-Glu-Gly-Glu-Leu-Glu-Gly-Thr-Thr-Thr-Lys (SEQ ID NO: 6) in which X stands for an amino acid residue which could not be determined,

2. for the 5110 and 38 kD bands (according to non-reducing SDS-PAGE):

Leu-Val-Pro-His-Leu-Gly-Asp-Arg-Glu (SEQ ID NO: 15)

3. for the 65 kD band (according to non-reducing SDS-PAGE)

In the N-terminal sequencing of the 65 kD band two parallel sequences were determined up to the 15th residue without interruption. Since one of the two sequences corresponded to a partial sequence of ubiquitin [36, 37], the following sequence was derived for the 65 kD band:

Leu-Pro-Ala-Gln-Val-Ala-Phe-X-Pro-Tyr-Ala-Pro-Glu-Pro-Gly-Ser-Thr-Cys. (SEQ ID NO: 16)

in which X stands for an amino acid residue which could not be determined.

Additional peptide sequences for 75(65) kDa-TNF-BP were determined:

Ile-X-Pro-Gly-Phe-Gly-Val-Ala-Tyr-Pro-Ala-Leu-Glu (SEQ ID NO: 11)

and

Ser-Gln-Leu-Glu-Thr-Pro-Glu-Thr-Leu-Leu-Gly-Ser-Thr-Glu-Glu-Lys-Pro-Leu (SEQ ID NO: 7) and Val-Phe-Cys-Thr (SEQ ID NO: 8)

and

Asn-Gln-Pro-Gln-Ala-Pro-Gly-Val-Glu-Ala-Ser-Gly-Ala-Gly-Glu-Ala (SEQ ID NO: 9) and Leu-Cys-Ala-Pro (SEQ ID NO: 12)

and

Val-Pro-His-Leu-Pro-Ala-Asp SEQ ID NO: 13)

and

Gly-Ser-Gln-Gly-Pro-Glu-Gln-Gln-X-X-Leu-Ile-X-Ala-Pro (SEQ ID NO: 14),

in which X stands for an amino acid residue which could not be determined.

Example 8

Determination of Base Sequences of Complementary DNA (cDNA)

Starting from the amino acid sequence according to formula IA there were synthesized having regard to the genetic code for the amino acid residues 2-7 and 17-23 corresponding completely degenerated oligonucleotides in suitable complementarity ("sense" and "antisense" oligonucleotides). Total cellular RNA was isolated from HL60 cells [42, 43] and the first cDNA strand was synthesized by oligo-dT priming or by priming with the "antisense" oligonucleotide using a cDNA synthesis kit (RPN 1256, Amersham, Amersham, England) according to the instructions of the manufacturer. This cDNA strand and the two synthesized degenerate "sense" and "antisense" oligonucleotides were used in a polymerase chain reaction (PCR, Perkin Elmer Cetus, Norwalk, Conn., USA according to the instructions of the manufacturer) to synthesize as a cDNA fragment the base sequence coding for the amino acid residues 8-16 (formula IA). The base sequence of this cDNA fragment accorded to: 5'-AGGGAGAAGAGAGATAGTGTGTGTCCC-3' (SEQ ID NO: 16). This cDNA fragment was used as a probe in order to identify according to a known procedure a cDNA clone coding for the 55 kD TNF-BP in a λgt11-cDNA gene bank from human placenta (42, 43). This clone was then cut according to usual methods from the λ-vector and cloned in the plasmids pUC18 (Pharmacia, Uppsala, Sweden) and pUC19 (Pharmacia, Uppsala, Sweden) and in the M13 mp18/M13 mp19 bacteriophage (Pharmacia, Uppsala, Sweden) (42, 43). The nucleotide sequence of this cDNA clone was determined using a Sequenase kit (U.S. Biochemical, Cleveland, Ohio, USA) according to the details of the manufacturer. The nucleotide sequence and the amino acid sequence derived therefrom for the 55 kD TNF-BP and its signal peptide (amino acid "−28" to amino acid "O") is given in FIG. **1** using the abbreviations for bases such as amino acids usual in the state of the art. From sequence comparisons with other already known receptor protein sequences there can be determined a N-terminal domain containing approximately 180 amino acids and a C-terminal domain containing 220 amino acids which are separated from one another by a transmembrane region of 19 amino acids (underlined in FIG. **1**) which is typical according to the sequence comparisons. Hypothetical glycosylation sites are characterized in FIG. **1** by asterisks above the corresponding amino acid.

Essentially analogous techniques were used to identify 75/65 kD TNF-BP-coding partial cDNA sequences, whereby, however, in this case genomic human DNA and completely degenerated 14-mer and 15-mer "sense" and "antisense" oligonucleotides derived from peptide IIA were used in order to produce a primary 26 by cDNA probe in a polymerase chain reaction. This cDNA probe was then used in a HL-60 cDNA library to identify cDNA clones of different lengths. This cDNA library was produced using isolated HL60 RNA and a cDNA cloning kit (Amersham) according to the details of the manufacturer. The sequence of such a cDNA clone is given in FIG. **4** (SEQ ID NO: 28), whereby repeated sequencing lead to the following correction as depicted in FIG. **6** (SEQ ID NO: 3). A threonine coded by "ACC" not "TCC", has to be at position 3 instead of the serine.

Example 9

Expression in COS1 Cells

Vectors starting from the plasmid "pN11" were constructed for the expression in COS cells. The plasmid "pN11" contains the efficient promotor and enhancer of the "major immediate-early" gene of human cytomegalovirus ("HCMV"; Boshart et al., Cell 41, 521-530, 1985). After the promotor there is situated a short DNA sequence which contains several restriction cleavage sites, which are present only once in the plasmid ("polylinker"), inter alia the cleavage sites for HindIII, BalI, BamHI and PvuII (see sequence).

PvuII

5'-AAGCTTGGCCAGGATCCAGCTGACT-GACTGATCGCGAGATC-3' (SEQ ID NO: 17)

3'-TTCGAACCGGTCCTAGGTCGACTGACT-GACTAGCGCTCTAG-5' (SEQ ID NO: 18)

After these cleavage sites there are situated three translation stop codons in all three reading frames. After the polylinker-sequence there is situated the 2nd intron and the polyadenylation signal of the preproinsulin gene of the rat (Lomedico et al., Cell 18, 545-558, 1979). The plasmid also contains the replication origin of the SV40 virus and a fragment from pBR322 which confers *E. coli*-bacteria ampicillin resistance and permits the replication of the plasmid in *E. coli*.

For the construction of the expression vector "pN123", this plasmid "pN11" was cleaved the restriction endonuclease PvuII and subsequently treated with alkaline phosphatase. The dephosphorylated vector was thereafter isolated from an agarose gel (V1). The 5'-projecting nucleotides of the EcoRI-cleaved 1.3 kb fragment of the 55 kD TNF-BP-cDNA (see Example 8) were filled in using Klenow enzyme. Subsequently, this fragment was isolated from an agarose gel (F1). Thereafter, V1 and F1 were joined together using T4-ligase. *E. coli* HB101 cells were then transformed with this ligation batch according to known methods [42]. By means of restriction analyses and DNA sequencing according to known methods [42] there were identified transformants which had been transformed with a plasmid and which contained the 1.3 kb EcoRI fragment of the 55 kD TNF-BP-cDNA in the correct orientation for expression via the HCMV-promoter. This vector received the designation "pN123".

The following procedure was used for the construction of the vector "pK19". A DNA fragment which contained only the cDNA coding for the extracellular part of the 55 kD TNF-BP (amino acids −28 to 182 according to FIG. **1**) was obtained by PCR technology (Saiki et al., Science 230, 1350-1354, 1985, see also Example 8). The following oligonucleotides were used in order to amplify the cDNA from "pN123" coding for the extracellular part of the 55 kD TNF-BP:

BAMHI

5'-CACAGGGATCCATAGCTGTCTG-GCATGGGCCTCTCCAC-3' (SEQ ID NO: 19)

ASP718

3'-CGTGACTCCTGAGTCCGTGGTGTAT-TATCTCTAGACCA TGGCCC-5' (SEQ ID NO: 20)

By means of these oligonucleotides there were also introduced two stop codons of the translation after amino acid 182. The thus-amplified DNA fragment was cleaved with BamHI and Asp718, the thereby resulting projecting ends were filled in using Klenow enzyme and this fragment was subsequently isolated from an agarose gel (F2). F2 was then ligated with V1 and the entire batch was used for the transformation of *E. coli* HB101, as already described. Transformants which had been transformed with a plasmid containing the DNA fragment in the correct orientation for the expression via the HCMV-

promoter were identified by DNA sequencing (see above). The plasmid isolated therefrom received the designation "pK19".

Transfection of the COS cells with the plasmids "pN123" or "pK19" was carried out according to the lipofection method published by Felgner et al. (Proc. Natl. Acad. Sci. USA 84, 7413-7417, 1987). 72 hours after the transfection had been effected the cells transfected with "pN123" were analyzed for binding with $^{125}$I-TNFα according to known methods. The results of the Scatchard analysis [Scatchard, G., Ann. N.Y. Acad. Sci. 51, 660, 1949] of the thus-obtained binding data (FIG. **2**A) is given in FIG. **2**B. The culture supernatants of the cells transfected with "pK19" were investigated in a "sandwich" test. For this purpose, PVC microtitre plates (Dynatech, Arlington, Va., USA) were sensitized with 100 μl/well of a rabbit-anti-mouse immunoglobulin (10 μg/ml PBS). Subsequently, the plates were washed and incubated (3 hours, 20° C.) with an anti-55 kD TNF-BP antibody which had been detected by its antigen binding and isolated according to Example 3, but which did not inhibit the TNF-binding to cells. The plates were then again washed and incubated overnight at 4° C. with 100 μl/well of the culture supernatant (diluted 1:4 with buffer A containing 1% skimmed milk powder: 50 mM Tris/HCl pH 7.4, 140 mM NaCl, 5 mM EDTA, 0.02% Na azide). The plates were emptied and incubated at 4° C. for 2 hours with buffer A containing $^{125}$I-TNFα ($10^6$ cpm/ml, 100 μl/well) with or without the addition of 2 μg/ml of unlabelled TNF. Thereafter, the plates were washed 4 times with PBS, the individual wells were cut out and measured in a λ-counter. The results of 5 parallel transfections (columns #2, 3, 4, 6 and 7), of two control transfections with the pN11 vector (columns #1, 5) and of a control with HL60 cell lysate (column #8) are given in FIG. **3**.

## Example 10

### Expression in Insect Cells

The plasmid "pVL941" (Luckow and Summers, 1989, "High Level Expression of Nonfused Foreign Genes with *Autographa californica* Nuclear Polyhedrosis virus Expression Vectors", Virology 170, 31-39) was used for the expression in a baculovirus expression system and was modified as follows. The single EcoRI restriction cleavage site in "pVL941" was removed by cleaving the plasmid with EcoRI and the projecting 5'-end was filled in with Klenow enzyme. The plasmid pVL941/E obtained therefrom was digested with BamHI and Asp718 and the vector trunk was subsequently isolated from an agarose gel. This fragment was ligated with a synthetic oligonucleotide of the following sequence:

BamHI EcoRI Asp718

5'-GATCCAGAATTCATAATAG-3' (SEQ ID NO: 21)

3'-GTCTTAAGTATTATCCATG-5' (SEQ ID NO: 22)

*E. coli* HB101 was transformed with the ligation batch and transformants containing a plasmid in which the oligonucleotide had been incorporated correctly were identified by restriction analysis and DNA sequencing according to known methods (see above); this plasmid was named "pNR704". For the construction of the transfer vector "pN113", this plasmid "pNR704" was cleaved with EcoRI, treated with alkaline phosphatase and the thus-produced vector trunk (V2) was subsequently isolated from an agarose gel. The 1.3 kb fragment of the 55 kD TNF-BP-cDNA cleaved with EcoRI as above was ligated with fragment V2. Transformants obtained with this ligation batch, which contained a plasmid containing the cDNA insert in the correct orientation for the expression via the polyhedron promoter, were identified (see above). The vector isolated therefrom received the designation "pN113".

The following procedure was used for the construction of the transfer vector "pN119". The 1.3 kb EcoRI/EcoRI fragment of the 55 kD TNF-BP cDNA in the "pUC19" plasmid (see Example 8) was digested with BanI and ligated with the following synthetic oligonucleotide:

BanI Asp718

5'-GCACCACATAATAGAGATCTGGTACCGGGAA-3' (SEQ ID NO: 23)

3'-GTGTATTATCTCTAGACCATGGCCC-5' (SEQ ID NO: 24)

Two stop codons of the translation after amino acid 182 and a cleavage site for the restriction endonuclease Asp718 are incorporated with the above adaptor. After carrying out ligation the batch was digested with EcoRI and Asp718 and the partial 55 kD TNF-BP fragment (F3) was isolated. Furthermore, the plasmid "pNR704", likewise cleaved with Asp718 and EcoRI, was ligated with F3 and the ligation batch was transformed into *E. coli* HB101. The identification of the transformants which contained a plasmid in which the partial 55 kD TNF-BP cDNA had been correctly integrated for the expression was effected as already described. The plasmid isolated from these transformants received the name "pN119".

The following procedure was used for the construction of the transfer vector "pN124". The cDNA fragment coding for the extracellular part of the 55 kD TNF-BP, described in Example 9, was amplified with the specified oligonucleotides with the aid of PCR technology as described in Example 9. This fragment was cleaved with BamHI and Asp718 and isolated from an agarose gel (F4). The plasmid "pNR704" was also cleaved with BamHI and Asp718 and the vector trunk (V4) was isolated (see above). The fragments V4 and F4 were ligated, *E. coli* HB101 was transformed therewith and the recombinant transfer vector "pN124" was identified and isolated as described.

The following procedure was used for the transfection of the insect cells. 3 μg of the transfer vector "pN113" were transfected with 1 μg of DNA of the *Autographa californica* nuclear polyhedrosisvirus (AcMNPV) (EP 127839) in Sf9 cells (ATCC CRL 1711). Polyhedron-negative viruses were identified and purified from "plaques" [52]. Sf9 cells were again infected with these recombinant viruses as described in [52]. After 3 days in the culture the infected cells were investigated for TNF-binding using $^{125}$I-TNFα. For this purpose, the transfected cells were washed from the cell culture dish with a Pasteur pipette and resuspended at a cell density of $5\times10^6$ cells/ml of culture medium [52] which contained 10 ng/ml of $^{125}$I-TNF-α, not only in the presence of, but also in the absence of 5 μg/ml of non-labelled TNF-α and incubated on ice for 2 hours. Thereafter, the cells were washed with pure culture medium and the cell-bound radioactivity was counted in a γ-counter (see Table 2).

TABLE 2

| Cells | Cell-bound radioactivity per $10^6$ cells |
|---|---|
| Non-infected cells (control) | 60 cpm |
| Infected cells | 1600 ± 330 cpm [1)] |

[1)] Average and standard deviation from 4 experiments

## Example 11

Analogously to the procedure described in Example 9, the cDNA fragment coding for the extracellular region of the 55

kDa TNF-BP was amplified in a polymerase chain reaction, but now using the following oligonucleotides as the primer:
Oligonucleotide 1:
Sst I
5'-TAC GAG CTC GGC CAT AGC TGT CTG GCA TG-3' (SEQ ID NO: 25)
Oligonucleotide 2:
Sst I
5'-ATA GAG CTC TGT GGT GCC TGA GTC CTC AG-3' (SEQ ID NO: 26)

This cDNA fragment was ligated in the pCD4-Hγ3 vector [DSM 5523; European Patent Application No. 90107393.2; Japanese Patent Application No. 108967/90; U.S. patent application Ser. No. 51/077,390] from which the CD4-cDNA had been removed via the SstI restriction cleavage sites. SstI cleavage sites are situated in vector pCD4-Hγ3 not only in front of, but also behind the CD4-partial sequence fragment. The construction was transfixed in J558 myeloma cells (ATCC No. TIB6) by means of protoplast fusion according to Oi et al. (Procd. Natl. Acad. Sci. USA 80, 825-829, 1983). Transfectants were selected by adding 5 μg/ml of mycophenolic acid and 250 μg/ml of xanthin (Traunecker et al., Eur. J. Immunol. 16, 851-854 [1986]) in basic medium (Dulbecco's modified Eagle's Medium, 10% foetal calf serum, $5\times10^{-5}$M 2-mercaptoethanol). The expression product secreted by the transfixed cells could be purified using usual methods of protein chemistry, e.g. TNF-BP-antibody affinity chromatography. Unless not already specifically indicated, standard procedures as described e.g. by Freshney, R. I. in "Culture of Animal Cells", Alan R. Liss, Inc., New York (1983) were used for the cultivation of the cell lines employed, for the cloning, for the selection or for the expansion of the cloned cells.

REFERENCES

1. G. E. Nedwin, S. L. Naylor, A. Y. Sakaguchi, D. Smith, J. Jarrett-Nedwin, D. Pennica, D. V. Goeddel and P. W. Gray: Nucl. Acids Res. 13, 6361, 1985
2. B. Beutler and A. Cerami: New England J. Med. 316, 379, 1987
3. L. J. Old: Science 230, 630, 1985
4. G. Trinchieri, M. Kobayashi, M. Rosen, R. Loudon, M. Murphy and B. Perussia: J. exp. Med. 164, 1206, 1986
5. J. Vilcek, V. J. Palombella, D. Henriksen-de Stefano, C. Swenson, R. Feinman, M. Hirai and M. Tsujimoto: J. exp. Med. 163, 632, 1986
6. B. J. Sugarman, B. B. Aggarwal, P. E. Hass, I. S. Figari, M. A. Palladino and H. M. Shepard: Science 230, 943, 1985
7. J. R. Gamble, J. M. Harlan, S. J. Klebanoff and M. A. Vadas: Proc. Natl. Acad. Sci. USA 82, 8667, 1985
8. N. Sato, T. Goto, K. Haranaka, N. Satomi, H. Nariuchi, Y. Mano and Y. Sawasaki: J. Natl. Cancer Inst. 76, 1113, 1986
9. A. H. Stolpen, E. C. Guinan, W. Fiers and J. S. Pober: Am. J. Pathol. 123, 16, 1986
10. J. S. Pober, L. A. Lapierre, A. H. Stolpen, T. A. Brock, T. A. Springer, W. Fiers, M. P. Bevilacqua, D. L. Mendrick and M. A Gimbrone: J. Immunol. 138, 3319, 1987
11. M. Kawakami, P. Pekala, M. Lane and A. Cerami: Proc. Natl. Acad. Sci. USA 79, 912, 1982
12. T. Collins, L. A. Lapierre, W. Fiers, J. L. Strominger and J. S Pober: Proc. Natl. Acad. Sci. USA 83, 446, 1986
13. G. H. W. Wong and D. V. Goeddel: Nature 323, 819, 1986
14. J. W. Lowenthal, D. W. Ballard, E. B•hnlein and W. C. Greene: Proc. Natl. Acad. Sci. USA 86, 2331, 1989
15. M. J. Lenardo, C. M. Fan, T. Maniatis and D. Baltimore: Cell 57, 287, 1989
16. A. E. Goldfeld and T. Maniatis: Proc. Natl. Acad. Sci. USA 86, 1490, 1989
17. A. Waage, A. Halsteuren and T. Espevik: Lancet, Febr. 14, 1987, 355,
18. C. O. Jacob and H. O. McDevitt: Nature 331, 356, 1988
19. G. E. Grau, L. F. Fajardo, P. Piquet, B. Allet, P. Lambert and P. Vassalli: Science 237, 1210, 1987
20. B. Beutler, I. W. Milsark and A. C. Cerami: Science 229, 869, 1985
21. B. B. Aggarwal, T. E. Eessalu and P. E. Hass: Nature 318, 665, 1985
22. M. Tsujimoto, Y. K. Yip and J. Vilcek: Proc. Natl. Acad. Sci. USA 82, 7626, 1985
23. C. Baglioni, S. McCandless, J. Tavernier and W. Fiers: J. Biol. Chem. 260, 13395, 1985
24. P. Hohmann, R. Remy, M. Brockhaus and A. P. G. M. van Loon: J. Biol. Chem., im Druck
25. F. C. Kull, S. Jacobs and P. Cuatrecasas: Proc. Natl. Acad. Sci. USA 82, 5756, 1985
26. A. A. Creasy, R. Yamamoto and Ch. R. Vitt: Proc. Natl. Acad. Sci. USA 84, 3293, 1987
27. G. B. Stauber, R. A. Aiyer and B. B. Aggarwal: J. Biol. Chem. 263, 19098, 1988
28. K. Hirano, K. Yamamoto, Y. Kobayashi and T. Osawa: J. Biochem. 105, 120, 1989
29. Y. Niitsu, N. Watanabe, H. Sone, H. Neda, N. Yamauchi, M. Maeda and I. Urushizaki: J. Biol. Resp. Modifiers 7, 276, 1988
30. I. Olsson, A. Grubb, U. Gullberg, M. Lantz, E. Nilsson, C. Peetre and H. Thysell: Abstract, 2nd Intern. Conference on Tumor Necrosis Factor and Related Cytokines, Napa, Calif., 15.-20. Januar 1989
31. H. R. Loetscher and M. Brockhaus: Abstract, 2nd Intern. Conference on Tumor Necrosis Factor and Related Cytokines, Napa, Calif., 15.-20. Januar 1989
32. M. Brockhaus, H. Loetscher, H.-P. Hohmann and W. Hunziker: Abstract, 2nd Intern. Conference on Tumor Necrosis Factor and Related Cytokines, Napa, Calif., 15.-20. Januar 1989
33. C. R. Cantor and P. R. Schimmel, in Biophysical Chemistry, W.H. Freeman, ed., San Francisco, 1980, P. 850
34. M. W. Hunkapiller, E. Lujan, F. Ostrander, L. E. Hood: Methods Enzymol. 91, 227, 1983
35. U. K. Lämmli: Nature 227, 680, 1970
36. T. St. John, W. M. Gallatin, M. Siegelman. H. T. Smith, V. A. Fried and I. L. Weissman: Science 231, 845, 1986
37. M. Siegelman, M. W. Bond, W. M. Gallatin, T. St. John, H. T. Smith, V. A. Fried and I. L. Weissman: Science 231, 823, 1986
38. H. Towbin, T. Staehelin and J. Gordon: Proc. Natl. Acad. Sci. USA 76, 4350, 1979
39. Dinarello, Ch. A., in Lymphokines, Vol. 14, E. Pick, ed., p. 1, Academic Press, London, 1987
40. D. J. Merchant, R. H. Kahn and W. H. Murphy: Handbook of Cell and Organ Culture, Burgess Publ. Co., Minneapolis, 1969
41. G. E. Grau, T. E. Taylor, M. E. Molyneux, J. J. Wirima, P. Vassalli, M. Hommel and P. Lambert: New Engl. J. Med. 320, 1586, 1989
42. J. Sambrook, E. F. Fritsch and T. Maniatis: Molecular. Cloning, A Laboratory Manual, 2nd ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, Cold Spring Harbor Laboratory Press, 1989
43. F. M. Ausubel, R. Brent, R. E. Kingston, D. D. Moore, J. A. Smith, J. G. Seidman and K. Struhl: Current Protocols in Molecular Biology 1987-1988, S. Wiley and Sons, New York, 1987

44. E. Harlow and D. Lane: Antibodies, A Laboratory Manual, Cold Spring Harbor Laboratory Publications, New York, 1988
45. S. Fazekas de St. Groth and D. Scheidegger: J. Immunol. Methods 35, 1, 1980
46. D. Pennica and D. V. Goeddel, in Lymphokines, Vol. 13, D. R. Webb and D. V. Goeddel, eds. p. 163, Academic Press, London, 1987
47. J. Tavernier, L. Franzen, A. Marmenout, J. van der Heyden, R. Muller, M. Ruysschaert, A. van Vliet, R. Banden and W. Fiers, in Lymphokines, Vol. 13, D. R. Webb and D. V. Goeddel, eds., p. 181, Academic Press, London
48. P. J. Fraker and J. C. Speck: Biochem. Biophys. Res. Commun. 80, 849, 1987
49. D. H. Erlich, D. H. Gelfand, R. K. Saiki: Nature 331, 61, 1988
50. Bosserhoff, J. Wallach and R. W. Frank: J. Chromatogr. 473, 71, 1989
51. R. Lathe: J. Mol. Biol. 183, 1, 1985
52. Luckow and Summers, "A Manual of Methods for Baculovirus Vectors and Insect Cell Culture Procedures", Texas Agricultural Experimental Station, Texas A & M University, Bulletin No. 1555, 2nd edition, 1988

SEQUENCE LISTING

<160> NUMBER OF SEQ ID NOS: 29

<210> SEQ ID NO 1
<211> LENGTH: 2111
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 1

gaattcgggg gggttcaaga tcactgggac caggccgtga tctctatgcc cgagtctcaa 60

ccctcaactg tcaccccaag gcacttggga cgtcctggac agaccgagtc ccgggaagcc 120

ccagcactgc cgctgccaca ctgccctgag cccaaatggg ggagtgagag gccatagctg 180

tctggcatgg gcctctccac cgtgcctgac ctgctgctgc cgctggtgct cctggagctg 240

ttggtgggaa tatacccctc aggggttatt ggactggtcc ctcacctagg ggacagggag 300

aagagagata gtgtgtgtcc ccaaggaaaa tatatccacc ctcaaaataa ttcgatttgc 360

tgtaccaagt gccacaaagg aacctacttg tacaatgact gtccaggccc ggggcaggat 420

acggactgca gggagtgtga gagcggctcc ttcaccgctt cagaaaacca cctcagacac 480

tgcctcagct gctccaaatg ccgaaaggaa atgggtcagg tggagatctc ttcttgcaca 540

gtggaccggg acaccgtgtg tggctgcagg aagaaccagt accggcatta ttggagtgaa 600

aacctttcc agtgcttcaa ttgcagcctc tgcctcaatg ggaccgtgca cctctcctgc 660

caggagaaac agaacaccgt gtgcacctgc catgcaggtt tctttctaag agaaaacgag 720

tgtgtctcct gtagtaactg taagaaaagc ctggagtgca cgaagttgtg cctaccccag 780

attgagaatg ttaagggcac tgaggactca ggcaccacag tgctgttgcc cctggtcatt 840

ttctttggtc tttgcctttt atccctcctc ttcattggtt taatgtatcg ctaccaacgg 900

tggaagtcca agctctactc cattgtttgt gggaaatcga cacctgaaaa agagggggag 960

cttgaaggaa ctactactaa gcccctggcc ccaaacccaa gcttcagtcc cactccaggc 1020

ttcaccccca ccctgggctt cagtcccgtg cccagttcca ccttcacctc cagctccacc 1080

tatacccccg gtgactgtcc caactttgcg gctccccgca gagaggtggc accaccctat 1140

caggggggctg accccatcct tgcgacagcc ctcgcctccg accccatccc caacccccctt 1200

cagaagtggg aggacagcgc ccacaagcca cagagcctag acactgatga ccccgcgacg 1260

ctgtacgccg tggtggagaa cgtgcccccg ttgcgctgga aggaattcgt gcggcgccta 1320

gggctgagcg accacgagat cgatcggctg gagctgcaga acgggcgctg cctgcgcgag 1380

gcgcaataca gcatgctggc gacctggagg cggcgcacgc cgcggcgcga ggccacgctg 1440

gagctgctgg gacgcgtgct ccgcgacatg gacctgctgg gctgcctgga ggacatcgag 1500

gaggcgcttt gcggccccgc cgccctcccg cccgcgccca gtcttctcag atgaggctgc 1560

-continued

gcccctgcgg gcagctctaa ggaccgtcct gcgagatcgc cttccaaccc cacttttttc 1620

tggaaaggag gggtcctgca ggggcaagca ggagctagca gccgcctact tggtgctaac 1680

ccctcgatgt acatagcttt tctcagctgc ctgcgcgccg ccgacagtca gcgctgtgcg 1740

cgcggagaga ggtgcgccgt gggctcaaga gcctgagtgg gtggtttgcg aggatgaggg 1800

acgctatgcc tcatgcccgt tttgggtgtc ctcaccagca aggctgctcg ggggcccctg 1860

gttcgtccct gagccttttt cacagtgcat aagcagtttt ttttgttttt gttttgtttt 1920

gttttgtttt taaatcaatc atgttacact aatagaaact tggcactcct gtgccctctg 1980

cctggacaag cacatagcaa gctgaactgt cctaaggcag gggcgagcac ggaacaatgg 2040

ggccttcagc tggagctgtg gacttttgta catacactaa aattctgaag ttaaaaaaaa 2100

aacccgaatt c 2111

<210> SEQ ID NO 2
<211> LENGTH: 455
<212> TYPE: PRT
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 2

Met Gly Leu Ser Thr Val Pro Asp Leu Leu Leu Pro Leu Val Leu Leu
1 5 10 15

Glu Leu Leu Val Gly Ile Tyr Pro Ser Gly Val Ile Gly Leu Val Pro
20 25 30

His Leu Gly Asp Arg Glu Lys Arg Asp Ser Val Cys Pro Gln Gly Lys
35 40 45

Tyr Ile His Pro Gln Asn Asn Ser Ile Cys Cys Thr Lys Cys His Lys
50 55 60

Gly Thr Tyr Leu Tyr Asn Asp Cys Pro Gly Pro Gly Gln Asp Thr Asp
65 70 75 80

Cys Arg Glu Cys Glu Ser Gly Ser Phe Thr Ala Ser Glu Asn His Leu
85 90 95

Arg His Cys Leu Ser Cys Ser Lys Cys Arg Lys Glu Met Gly Gln Val
100 105 110

Glu Ile Ser Ser Cys Thr Val Asp Arg Asp Thr Val Cys Gly Cys Arg
115 120 125

Lys Asn Gln Tyr Arg His Tyr Trp Ser Glu Asn Leu Phe Gln Cys Phe
130 135 140

Asn Cys Ser Leu Cys Leu Asn Gly Thr Val His Leu Ser Cys Gln Glu
145 150 155 160

Lys Gln Asn Thr Val Cys Thr Cys His Ala Gly Phe Phe Leu Arg Glu
165 170 175

Asn Glu Cys Val Ser Cys Ser Asn Cys Lys Lys Ser Leu Glu Cys Thr
180 185 190

Lys Leu Cys Leu Pro Gln Ile Glu Asn Val Lys Gly Thr Glu Asp Ser
195 200 205

Gly Thr Thr Val Leu Leu Pro Leu Val Ile Phe Phe Gly Leu Cys Leu
210 215 220

Leu Ser Leu Leu Phe Ile Gly Leu Met Tyr Arg Tyr Gln Arg Trp Lys
225 230 235 240

Ser Lys Leu Tyr Ser Ile Val Cys Gly Lys Ser Thr Pro Glu Lys Glu
245 250 255

Gly Glu Leu Glu Gly Thr Thr Thr Lys Pro Leu Ala Pro Asn Pro Ser
260 265 270

Phe Ser Pro Thr Pro Gly Phe Thr Pro Thr Leu Gly Phe Ser Pro Val

-continued

```
        275                 280                 285

Pro Ser Ser Thr Phe Thr Ser Ser Ser Thr Tyr Thr Pro Gly Asp Cys
    290                 295                 300

Pro Asn Phe Ala Ala Pro Arg Arg Glu Val Ala Pro Pro Tyr Gln Gly
305                 310                 315                 320

Ala Asp Pro Ile Leu Ala Thr Ala Leu Ala Ser Asp Pro Ile Pro Asn
                325                 330                 335

Pro Leu Gln Lys Trp Glu Asp Ser Ala His Lys Pro Gln Ser Leu Asp
            340                 345                 350

Thr Asp Asp Pro Ala Thr Leu Tyr Ala Val Val Glu Asn Val Pro Pro
        355                 360                 365

Leu Arg Trp Lys Glu Phe Val Arg Arg Leu Gly Leu Ser Asp His Glu
    370                 375                 380

Ile Asp Arg Leu Glu Leu Gln Asn Gly Arg Cys Leu Arg Glu Ala Gln
385                 390                 395                 400

Tyr Ser Met Leu Ala Thr Trp Arg Arg Arg Thr Pro Arg Arg Glu Ala
                405                 410                 415

Thr Leu Glu Leu Leu Gly Arg Val Leu Arg Asp Met Asp Leu Leu Gly
            420                 425                 430

Cys Leu Glu Asp Ile Glu Glu Ala Leu Cys Gly Pro Ala Ala Leu Pro
        435                 440                 445

Pro Ala Pro Ser Leu Leu Arg
    450                 455


<210> SEQ ID NO 3
<211> LENGTH: 2339
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 3

tcggacaccg tgtgtgactc ctgtgaggac agcacataca cccagctctg gaactgggtt     60

cccgagtgct tgagctgtgg ctcccgctgt agctctgacc aggtggaaac tcaagcctgc    120

actcgggaac agaaccgcat ctgcacctgc aggcccggct ggtactgcgc gctgagcaag    180

caggaggggt gccggctgtg cgcgccgctg ccgaagtgcc gcccgggctt cggcgtggcc    240

agaccaggaa ctgaaacatc agacgtggtg tgcaagccct gtgccccggg gacgttctcc    300

aacacgactt catccacgga tatttgcagg ccccaccaga tctgtaacgt ggtggccatc    360

cctgggaatg caagcaggga tgcagtctgc acgtccacgt cccccacccg gagtatggcc    420

ccaggggcag tacacttacc ccagccagtg tccacacgat cccaacacac gcagccaagt    480

ccagaaccca gcactgctcc aagcacctcc ttcctgctcc caatgggccc cagcccccca    540

gctgaaggga gcactggcga cttcgctctt ccagttggac tgattgtggg tgtgacagcc    600

ttgggtctac taataatagg agtggtgaac tgtgtcatca tgacccaggt gaaaaagaag    660

cccttgtgcc tgcagagaga agccaaggtg cctcacttgc ctgccgataa ggcccggggt    720

acacagggcc ccgagcagca gcacctgctg atcacagcgc cgagctccag cagcagctcc    780

ctggagagct cggccagtgc gttggacaga agggcgccca ctcggaacca gccacaggca    840

ccaggcgtgg aggccagtgg ggccggggag gcccgggcca gcaccgggag ctcagcagat    900

tcttcccctg gtggccatgg gacccaggtc aatgtcacct gcatcgtgaa cgtctgtagc    960

agctctgacc acagctcaca gtgctcctcc caagccagct ccacaatggg agacacagat   1020

tccagccccc cggagtcccc gaaggacgag caggtcccct tctccaagga ggaatgtgcc   1080

tttcggtcac agctggagac gccagagacc ctgctgggga gcaccgaaga gaagcccctg   1140
```

```
ccccttggag tgcctgatgc tgggatgaag cccagttaac caggccggtg tgggctgtgt   1200

cgtagccaag gtggctgagc cctggcagga tgaccctgcg aaggggccct ggtccttcca   1260

ggcccccacc actaggactc tgaggctctt tctgggccaa gttcctctag tgccctccac   1320

agccgcagcc tccctctgac ctgcaggcca agagcagagg cagcgagttg tggaaagcct   1380

ctgctgccat ggcgtgtccc tctcggaagg ctggctgggc atggacgttc ggggcatgct   1440

ggggcaagtc cctgagtctc tgtgacctgc cccgcccagc tgcacctgcc agcctggctt   1500

ctggagccct tgggtttttt gtttgtttgt ttgtttgttt gtttgtttct ccccctgggc   1560

tctgcccagc tctggcttcc agaaaacccc agcatccttt tctgcagagg ggctttctgg   1620

agaggaggga tgctgcctga gtcacccatg aagacaggac agtgcttcag cctgaggctg   1680

agactgcggg atggtcctgg ggctctgtgc agggaggagg tggcagccct gtagggaacg   1740

gggtccttca agttagctca ggaggcttgg aaagcatcac ctcaggccag gtgcagtggc   1800

tcacgcctat gatcccagca ctttgggagg ctgaggcggg tggatcacct gaggttagga   1860

gttcgagacc agcctggcca acatggtaaa accccatctc tactaaaaat acagaaatta   1920

gccgggcgtg gtggcgggca cctatagtcc cagctactca gaagcctgag gctgggaaat   1980

cgtttgaacc cgggaagcgg aggttgcagg gagccgagat cacgccactg cactccagcc   2040

tgggcgacag agcgagagtc tgtctcaaaa gaaaaaaaaa aagcaccgcc tccaaatgct   2100

aacttgtcct tttgtaccat ggtgtgaaag tcagatgccc agagggccca ggcaggccac   2160

catattcagt gctgtggcct gggcaagata acgcacttct aactagaaat ctgccaattt   2220

tttaaaaaag taagtaccac tcaggccaac aagccaacga caaagccaaa ctctgccagc   2280

cacatccaac cccccacctg ccatttgcac cctccgcctt cactccggtg tgcctgcag    2339
```

<210> SEQ ID NO 4
<211> LENGTH: 392
<212> TYPE: PRT
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 4

```
Ser Asp Thr Val Cys Asp Ser Cys Glu Asp Ser Thr Tyr Thr Gln Leu
1               5                   10                  15

Trp Asn Trp Val Pro Glu Cys Leu Ser Cys Gly Ser Arg Cys Ser Ser
            20                  25                  30

Asp Gln Val Glu Thr Gln Ala Cys Thr Arg Glu Gln Asn Arg Ile Cys
        35                  40                  45

Thr Cys Arg Pro Gly Trp Tyr Cys Ala Leu Ser Lys Gln Glu Gly Cys
    50                  55                  60

Arg Leu Cys Ala Pro Leu Pro Lys Cys Arg Pro Gly Phe Gly Val Ala
65                  70                  75                  80

Arg Pro Gly Thr Glu Thr Ser Asp Val Val Cys Lys Pro Cys Ala Pro
                85                  90                  95

Gly Thr Phe Ser Asn Thr Thr Ser Ser Thr Asp Ile Cys Arg Pro His
            100                 105                 110

Gln Ile Cys Asn Val Val Ala Ile Pro Gly Asn Ala Ser Arg Asp Ala
        115                 120                 125

Val Cys Thr Ser Thr Ser Pro Thr Arg Ser Met Ala Pro Gly Ala Val
    130                 135                 140

His Leu Pro Gln Pro Val Ser Thr Arg Ser Gln His Thr Gln Pro Ser
145                 150                 155                 160

Pro Glu Pro Ser Thr Ala Pro Ser Thr Ser Phe Leu Leu Pro Met Gly
```

```
                165                 170                 175

Pro Ser Pro Pro Ala Glu Gly Ser Thr Gly Asp Phe Ala Leu Pro Val
            180                 185                 190

Gly Leu Ile Val Gly Val Thr Ala Leu Gly Leu Leu Ile Ile Gly Val
        195                 200                 205

Val Asn Cys Val Ile Met Thr Gln Val Lys Lys Lys Pro Leu Cys Leu
    210                 215                 220

Gln Arg Glu Ala Lys Val Pro His Leu Pro Ala Asp Lys Ala Arg Gly
225                 230                 235                 240

Thr Gln Gly Pro Glu Gln Gln His Leu Leu Ile Thr Ala Pro Ser Ser
                245                 250                 255

Ser Ser Ser Ser Leu Glu Ser Ser Ala Ser Ala Leu Asp Arg Arg Ala
            260                 265                 270

Pro Thr Arg Asn Gln Pro Gln Ala Pro Gly Val Glu Ala Ser Gly Ala
        275                 280                 285

Gly Glu Ala Arg Ala Ser Thr Gly Ser Ser Ala Asp Ser Ser Pro Gly
    290                 295                 300

Gly His Gly Thr Gln Val Asn Val Thr Cys Ile Val Asn Val Cys Ser
305                 310                 315                 320

Ser Ser Asp His Ser Ser Gln Cys Ser Ser Gln Ala Ser Ser Thr Met
                325                 330                 335

Gly Asp Thr Asp Ser Ser Pro Ser Glu Ser Pro Lys Asp Glu Gln Val
            340                 345                 350

Pro Phe Ser Lys Glu Glu Cys Ala Phe Arg Ser Gln Leu Glu Thr Pro
        355                 360                 365

Glu Thr Leu Leu Gly Ser Thr Glu Glu Lys Pro Leu Pro Leu Gly Val
    370                 375                 380

Pro Asp Ala Gly Met Lys Pro Ser
385                 390


<210> SEQ ID NO 5
<211> LENGTH: 28
<212> TYPE: PRT
<213> ORGANISM: Artificial sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic peptide
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (25)..(25)
<223> OTHER INFORMATION: Xaa = unknown amino acid

<400> SEQUENCE: 5

Leu Val Pro His Leu Gly Asp Arg Glu Lys Arg Asp Ser Val Cys Pro
1               5                   10                  15

Gln Gly Lys Tyr Ile His Pro Gln Xaa Asn Ser Ile
            20                  25


<210> SEQ ID NO 6
<211> LENGTH: 15
<212> TYPE: PRT
<213> ORGANISM: Artificial sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic peptide

<400> SEQUENCE: 6

Ser Thr Pro Glu Lys Glu Gly Glu Leu Glu Gly Thr Thr Thr Lys
1               5                   10                  15


<210> SEQ ID NO 7
<211> LENGTH: 18
```

<212> TYPE: PRT
<213> ORGANISM: Artificial sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic peptide

<400> SEQUENCE: 7

Ser Gln Leu Glu Thr Pro Glu Thr Leu Leu Gly Ser Thr Glu Glu Lys
1 5 10 15

Pro Leu

<210> SEQ ID NO 8
<211> LENGTH: 4
<212> TYPE: PRT
<213> ORGANISM: Artificial sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic peptide

<400> SEQUENCE: 8

Val Phe Cys Thr
1

<210> SEQ ID NO 9
<211> LENGTH: 16
<212> TYPE: PRT
<213> ORGANISM: Artificial sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic peptide

<400> SEQUENCE: 9

Asn Gln Pro Gln Ala Pro Gly Val Glu Ala Ser Gly Ala Gly Glu Ala
1 5 10 15

<210> SEQ ID NO 10
<211> LENGTH: 18
<212> TYPE: PRT
<213> ORGANISM: Artificial sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic peptide
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (8)..(8)
<223> OTHER INFORMATION: Xaa = unknown amino acid

<400> SEQUENCE: 10

Leu Pro Ala Gln Val Ala Phe Xaa Pro Tyr Ala Pro Glu Pro Gly Ser
1 5 10 15

Thr Cys

<210> SEQ ID NO 11
<211> LENGTH: 13
<212> TYPE: PRT
<213> ORGANISM: Artificial sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic peptide
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (2)..(2)
<223> OTHER INFORMATION: Xaa = unknown amino acid

<400> SEQUENCE: 11

Ile Xaa Pro Gly Phe Gly Val Ala Tyr Pro Ala Leu Glu
1 5 10

<210> SEQ ID NO 12
<211> LENGTH: 4
<212> TYPE: PRT
<213> ORGANISM: Artificial sequence
<220> FEATURE:

<223> OTHER INFORMATION: Synthetic peptide

<400> SEQUENCE: 12

Leu Cys Ala Pro
1

<210> SEQ ID NO 13
<211> LENGTH: 7
<212> TYPE: PRT
<213> ORGANISM: Artificial sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic peptide

<400> SEQUENCE: 13

Val Pro His Leu Pro Ala Asp
1 5

<210> SEQ ID NO 14
<211> LENGTH: 15
<212> TYPE: PRT
<213> ORGANISM: Artificial sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic peptide
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (9)..(10)
<223> OTHER INFORMATION: Xaa = unknown amino acid
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (13)..(13)
<223> OTHER INFORMATION: Xaa = unknown amino acid

<400> SEQUENCE: 14

Gly Ser Gln Gly Pro Glu Gln Gln Xaa Xaa Leu Ile Xaa Ala Pro
1 5 10 15

<210> SEQ ID NO 15
<211> LENGTH: 9
<212> TYPE: PRT
<213> ORGANISM: Artificial sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic peptide

<400> SEQUENCE: 15

Leu Val Pro His Leu Gly Asp Arg Glu
1 5

<210> SEQ ID NO 16
<211> LENGTH: 27
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic primer

<400> SEQUENCE: 16

agggagaaga gagatagtgt gtgtccc 27

<210> SEQ ID NO 17
<211> LENGTH: 41
<212> TYPE: DNA
<213> ORGANISM: Artificial sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic primer

<400> SEQUENCE: 17

aagcttggcc aggatccagc tgactgactg atcgcgagat c 41

<210> SEQ ID NO 18

<211> LENGTH: 41
<212> TYPE: DNA
<213> ORGANISM: Artificial sequence
<220> FEATURE:
<223> OTHER INFORMATION: Antisense primer

<400> SEQUENCE: 18

gatctcgcga tcagtcagtc agctggatcc tggccaagct t 41

<210> SEQ ID NO 19
<211> LENGTH: 38
<212> TYPE: DNA
<213> ORGANISM: Artificial sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic primer

<400> SEQUENCE: 19

cacagggatc catagctgtc tggcatgggc ctctccac 38

<210> SEQ ID NO 20
<211> LENGTH: 44
<212> TYPE: DNA
<213> ORGANISM: Artificial sequence
<220> FEATURE:
<223> OTHER INFORMATION: Antisense primer

<400> SEQUENCE: 20

cccggtacca gatctctatt atgtggtgcc tgagtcctca gtgc 44

<210> SEQ ID NO 21
<211> LENGTH: 19
<212> TYPE: DNA
<213> ORGANISM: Artificial sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic primer

<400> SEQUENCE: 21

gatccagaat tcataatag 19

<210> SEQ ID NO 22
<211> LENGTH: 19
<212> TYPE: DNA
<213> ORGANISM: Artificial sequence
<220> FEATURE:
<223> OTHER INFORMATION: Antisense primer

<400> SEQUENCE: 22

gtacctatta tgaattctg 19

<210> SEQ ID NO 23
<211> LENGTH: 31
<212> TYPE: DNA
<213> ORGANISM: Artificial sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic primer

<400> SEQUENCE: 23

gcaccacata tagagatct ggtaccggga a 31

<210> SEQ ID NO 24
<211> LENGTH: 25
<212> TYPE: DNA
<213> ORGANISM: Artificial sequence
<220> FEATURE:
<223> OTHER INFORMATION: Antisense primer

<400> SEQUENCE: 24

cccggtacca gatctctatt atgtg 25

<210> SEQ ID NO 25
<211> LENGTH: 29
<212> TYPE: DNA
<213> ORGANISM: Artificial sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic primer

<400> SEQUENCE: 25

tacgagctcg gccatagctg tctggcatg 29

<210> SEQ ID NO 26
<211> LENGTH: 29
<212> TYPE: DNA
<213> ORGANISM: Artificial sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic primer

<400> SEQUENCE: 26

atagagctct gtggtgcctg agtcctcag 29

<210> SEQ ID NO 27
<211> LENGTH: 461
<212> TYPE: PRT
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 27

Met Ala Pro Val Ala Val Trp Ala Ala Leu Ala Val Gly Leu Glu Leu
1 5 10 15

Trp Ala Ala Ala His Ala Leu Pro Ala Gln Val Ala Phe Thr Pro Tyr
20 25 30

Ala Pro Glu Pro Gly Ser Thr Cys Arg Leu Arg Glu Tyr Tyr Asp Gln
35 40 45

Thr Ala Gln Met Cys Cys Ser Lys Cys Ser Pro Gly Gln His Ala Lys
50 55 60

Val Phe Cys Thr Lys Thr Ser Asp Thr Val Cys Asp Ser Cys Glu Asp
65 70 75 80

Ser Thr Tyr Thr Gln Leu Trp Asn Trp Val Pro Glu Cys Leu Ser Cys
85 90 95

Gly Ser Arg Cys Ser Ser Asp Gln Val Glu Thr Gln Ala Cys Thr Arg
100 105 110

Glu Gln Asn Arg Ile Cys Thr Cys Arg Pro Gly Trp Tyr Cys Ala Leu
115 120 125

Ser Lys Gln Glu Gly Cys Arg Leu Cys Ala Pro Leu Arg Lys Cys Arg
130 135 140

Pro Gly Phe Gly Val Ala Arg Pro Gly Thr Glu Thr Ser Asp Val Val
145 150 155 160

Cys Lys Pro Cys Ala Pro Gly Thr Phe Ser Asn Thr Thr Ser Ser Thr
165 170 175

Asp Ile Cys Arg Pro His Gln Ile Cys Asn Val Val Ala Ile Pro Gly
180 185 190

Asn Ala Ser Met Asp Ala Val Cys Thr Ser Thr Ser Pro Thr Arg Ser
195 200 205

Met Ala Pro Gly Ala Val His Leu Pro Gln Pro Val Ser Thr Arg Ser
210 215 220

Gln His Thr Gln Pro Thr Pro Glu Pro Ser Thr Ala Pro Ser Thr Ser
225 230 235 240

Phe Leu Leu Pro Met Gly Pro Ser Pro Pro Ala Glu Gly Ser Thr Gly

-continued

```
               245                 250                 255

Asp Phe Ala Leu Pro Val Gly Leu Ile Val Gly Val Thr Ala Leu Gly
            260                 265                 270

Leu Leu Ile Ile Gly Val Val Asn Cys Val Ile Met Thr Gln Val Lys
        275                 280                 285

Lys Lys Pro Leu Cys Leu Gln Arg Glu Ala Lys Val Pro His Leu Pro
    290                 295                 300

Ala Asp Lys Ala Arg Gly Thr Gln Gly Pro Glu Gln Gln His Leu Leu
305                 310                 315                 320

Ile Thr Ala Pro Ser Ser Ser Ser Ser Ser Leu Glu Ser Ser Ala Ser
                325                 330                 335

Ala Leu Asp Arg Arg Ala Pro Thr Arg Asn Gln Pro Gln Ala Pro Gly
            340                 345                 350

Val Glu Ala Ser Gly Ala Gly Glu Ala Arg Ala Ser Thr Gly Ser Ser
        355                 360                 365

Asp Ser Ser Pro Gly Gly His Gly Thr Gln Val Asn Val Thr Cys Ile
    370                 375                 380

Val Asn Val Cys Ser Ser Ser Asp His Ser Ser Gln Cys Ser Ser Gln
385                 390                 395                 400

Ala Ser Ser Thr Met Gly Asp Thr Asp Ser Ser Pro Ser Glu Ser Pro
                405                 410                 415

Lys Asp Glu Gln Val Pro Phe Ser Lys Glu Glu Cys Ala Phe Arg Ser
            420                 425                 430

Gln Leu Glu Thr Pro Glu Thr Leu Leu Gly Ser Thr Glu Glu Lys Pro
        435                 440                 445

Leu Pro Leu Gly Val Pro Asp Ala Gly Met Lys Pro Ser
    450                 455                 460
```

<210> SEQ ID NO 28
<211> LENGTH: 2339
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 28

```
tcggactccg tgtgtgactc ctgtgaggac agcacataca cccagctctg gaactgggtt     60

cccgagtgct tgagctgtgg ctcccgctgt agctctgacc aggtggaaac tcaagcctgc    120

actcgggaac agaaccgcat ctgcacctgc aggcccggct ggtactgcgc gctgagcaag    180

caggaggggt gccggctgtg cgcgccgctg ccgaagtgcc gcccgggctt cggcgtggcc    240

agaccaggaa ctgaaacatc agacgtggtg tgcaagccct gtgccccggg gacgttctcc    300

aacacgactt catccacgga tatttgcagg ccccaccaga tctgtaacgt ggtggccatc    360

cctgggaatg caagcaggga tgcagtctgc acgtccacgt cccccacccg gagtatggcc    420

ccaggggcag tacacttacc ccagccagtg tccacacgat cccaacacac gcagccaagt    480

ccagaaccca gcactgctcc aagcacctcc ttcctgctcc caatgggccc cagcccccca    540

gctgaaggga gcactggcga cttcgctctt ccagttggac tgattgtggg tgtgacagcc    600

ttgggtctac taataatagg agtggtgaac tgtgtcatca tgacccaggt gaaaaagaag    660

cccttgtgcc tgcagagaga agccaaggtg cctcacttgc ctgccgataa ggcccggggt    720

acacagggcc ccgagcagca gcacctgctg atcacagcgc cgagctccag cagcagctcc    780

ctggagagct cggccagtgc gttggacaga agggcgccca ctcggaacca gccacaggca    840

ccaggcgtgg aggccagtgg ggccggggag gcccgggcca gcaccgggag ctcagcagat    900

tcttcccctg gtggccatgg gacccaggtc aatgtcacct gcatcgtgaa cgtctgtagc    960
```

-continued

```
agctctgacc acagctcaca gtgctcctcc caagccagct ccacaatggg agacacagat   1020

tccagcccct cggagtcccc gaaggacgag caggtcccct tctccaagga ggaatgtgcc   1080

tttcggtcac agctggagac gccagagacc ctgctgggga gcaccgaaga gaagcccctg   1140

ccccttggag tgcctgatgc tgggatgaag cccagttaac caggccggtg tgggctgtgt   1200

cgtagccaag gtggctgagc cctggcagga tgaccctgcg aaggggccct ggtccttcca   1260

ggcccccacc actaggactc tgaggctctt tctgggccaa gttcctctag tgccctccac   1320

agccgcagcc tccctctgac ctgcaggcca agagcagagg cagcgagttg tggaaagcct   1380

ctgctgccat ggcgtgtccc tctcggaagg ctggctgggc atggacgttc ggggcatgct   1440

ggggcaagtc cctgagtctc tgtgacctgc cccgcccagc tgcacctgcc agcctggctt   1500

ctggagccct tgggtttttt gtttgtttgt ttgtttgttt gtttgtttct ccccctgggc   1560

tctgcccagc tctggcttcc agaaaacccc agcatccttt tctgcagagg ggctttctgg   1620

agaggaggga tgctgcctga gtcacccatg aagacaggac agtgcttcag cctgaggctg   1680

agactgcggg atggtcctgg ggctctgtgc agggaggagg tggcagccct gtagggaacg   1740

gggtccttca agttagctca ggaggcttgg aaagcatcac ctcaggccag gtgcagtggc   1800

tcacgcctat gatcccagca ctttgggagg ctgaggcggg tggatcacct gaggttagga   1860

gttcgagacc agcctggcca acatggtaaa accccatctc tactaaaaat acagaaatta   1920

gccgggcgtg gtggcgggca cctatagtcc cagctactca gaagcctgag gctgggaaat   1980

cgtttgaacc cgggaagcgg aggttgcagg gagccgagat cacgccactg cactccagcc   2040

tgggcgacag agcgagagtc tgtctcaaaa gaaaaaaaaa aagcaccgcc tccaaatgct   2100

aacttgtcct tttgtaccat ggtgtgaaag tcagatgccc agagggccca ggcaggccac   2160

catattcagt gctgtggcct gggcaagata acgcacttct aactagaaat ctgccaattt   2220

tttaaaaaag taagtaccac tcaggccaac aagccaacga caaagccaaa ctctgccagc   2280

cacatccaac cccccacctg ccatttgcac cctccgcctt cactccggtg tgcctgcag    2339
```

<210> SEQ ID NO 29
<211> LENGTH: 392
<212> TYPE: PRT
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 29

```
Ser Asp Ser Val Cys Asp Ser Cys Glu Asp Ser Thr Tyr Thr Gln Leu
1               5                   10                  15

Trp Asn Trp Val Pro Glu Cys Leu Ser Cys Gly Ser Arg Cys Ser Ser
            20                  25                  30

Asp Gln Val Glu Thr Gln Ala Cys Thr Arg Glu Gln Asn Arg Ile Cys
        35                  40                  45

Thr Cys Arg Pro Gly Trp Tyr Cys Ala Leu Ser Lys Gln Glu Gly Cys
    50                  55                  60

Arg Leu Cys Ala Pro Leu Pro Lys Cys Arg Pro Gly Phe Gly Val Ala
65                  70                  75                  80

Arg Pro Gly Thr Glu Thr Ser Asp Val Val Cys Lys Pro Cys Ala Pro
                85                  90                  95

Gly Thr Phe Ser Asn Thr Thr Ser Ser Thr Asp Ile Cys Arg Pro His
            100                 105                 110

Gln Ile Cys Asn Val Val Ala Ile Pro Gly Asn Ala Ser Arg Asp Ala
        115                 120                 125

Val Cys Thr Ser Thr Ser Pro Thr Arg Ser Met Ala Pro Gly Ala Val
```

-continued

```
    130                 135                 140

His Leu Pro Gln Pro Val Ser Thr Arg Ser Gln His Thr Gln Pro Ser
145                 150                 155                 160

Pro Glu Pro Ser Thr Ala Pro Ser Thr Ser Phe Leu Leu Pro Met Gly
                165                 170                 175

Pro Ser Pro Pro Ala Glu Gly Ser Thr Gly Asp Phe Ala Leu Pro Val
            180                 185                 190

Gly Leu Ile Val Gly Val Thr Ala Leu Gly Leu Leu Ile Ile Gly Val
        195                 200                 205

Val Asn Cys Val Ile Met Thr Gln Val Lys Lys Lys Pro Leu Cys Leu
    210                 215                 220

Gln Arg Glu Ala Lys Val Pro His Leu Pro Ala Asp Lys Ala Arg Gly
225                 230                 235                 240

Thr Gln Gly Pro Glu Gln Gln His Leu Leu Ile Thr Ala Pro Ser Ser
                245                 250                 255

Ser Ser Ser Ser Leu Glu Ser Ser Ala Ser Ala Leu Asp Arg Arg Ala
            260                 265                 270

Pro Thr Arg Asn Gln Pro Gln Ala Pro Gly Val Glu Ala Ser Gly Ala
        275                 280                 285

Gly Glu Ala Arg Ala Ser Thr Gly Ser Ser Ala Asp Ser Ser Pro Gly
    290                 295                 300

Gly His Gly Thr Gln Val Asn Val Thr Cys Ile Val Asn Val Cys Ser
305                 310                 315                 320

Ser Ser Asp His Ser Ser Gln Cys Ser Ser Gln Ala Ser Ser Thr Met
                325                 330                 335

Gly Asp Thr Asp Ser Ser Pro Ser Glu Ser Pro Lys Asp Glu Gln Val
            340                 345                 350

Pro Phe Ser Lys Glu Glu Cys Ala Phe Arg Ser Gln Leu Glu Thr Pro
        355                 360                 365

Glu Thr Leu Leu Gly Ser Thr Glu Glu Lys Pro Leu Pro Leu Gly Val
    370                 375                 380

Pro Asp Ala Gly Met Lys Pro Ser
385                 390
```

The invention claimed is:

**1**. A method comprising the steps of:

(a) culturing a host cell comprising a polynucleotide, wherein the polynucleotide encodes a protein consisting of:

(i) the extracellular region of an insoluble human TNF receptor, wherein the insoluble human TNF receptor has an apparent molecular weight of about 75 kilodaltons as determined on a non-reducing SDS-polyacrylamide gel and comprises the amino acid sequence LPAQVAFX-PYAPEPGSTC (SEQ ID NO: 10), and

(ii) all of the domains of the constant region of a human IgG immunoglobulin heavy chain other than the first domain of said constant region, and

(b) purifying an expression product of the polynucleotide from the cell mass or the culture medium.

**2**. The method of claim **1**, wherein the host cell is a CHO cell.

**3**. The method of claim **1**, wherein the IgG heavy chain is an $IgG_1$ heavy chain.

**4**. A polynucleotide encoding a protein consisting of:

(a) the extracellular region of an insoluble human TNF receptor,

wherein the insoluble human TNF receptor (i) has an apparent molecular weight of about 75 kilodaltons as determined on a non-reducing SDS-polyacrylamide gel and (ii) comprises the amino acid sequence LPAQVAFXPYAPEPGSTC (SEQ ID NO: 10), and

(b) all of the domains of the constant region of a human $IgG_1$ immunoglobulin heavy chain other than the first domain of said constant region.

**5**. A vector comprising the polynucleotide of claim **4**.

**6**. A mammalian host cell comprising the polynucleotide of claim **4**.

**7**. A method comprising the steps of:

(a) culturing a host cell comprising a polynucleotide, wherein the polynucleotide encodes a protein consisting of:

(i) the extracellular region of an insoluble human TNF receptor, wherein the insoluble human TNF receptor comprises the amino acid sequence of SEQ ID NO:27 and

(ii) all of the domains of the constant region of a human IgG immunoglobulin heavy chain other than the first domain of said constant region, and

(b) purifying an expression product of the polynucleotide from the cell mass or the culture medium.

**8**. The method of claim **7**, wherein the human IgG immunoglobulin heavy chain is an $IgG_1$ heavy chain.

**9**. The method of claim **7**, wherein the host cell is a CHO cell.

**10**. The method of claim **8**, wherein the host cell is a CHO cell.

* * * * *

# EXHIBIT 3

US006872549B2

(12) **United States Patent**
**Van Ness et al.**

(10) **Patent No.: US 6,872,549 B2**
(45) **Date of Patent: Mar. 29, 2005**

(54) **METHODS FOR INCREASING POLYPEPTIDE PRODUCTION**

(75) Inventors: **Kirk P. Van Ness**, Seattle, WA (US); **Michael T. Trentalange**, Seattle, WA (US); **Bradley D. Dell**, Seattle, WA (US); **Jeffrey T. McGrew**, Seattle, WA (US)

(73) Assignee: **Immunex Corporation**, Thousand Oaks, CA (US)

( * ) Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 48 days.

(21) Appl. No.: **10/400,334**

(22) Filed: **Mar. 27, 2003**

(65) **Prior Publication Data**

US 2003/0211579 A1 Nov. 13, 2003

**Related U.S. Application Data**

(60) Provisional application No. 60/368,246, filed on Mar. 27, 2002, and provisional application No. 60/368,248, filed on Mar. 27, 2002.

(51) **Int. Cl.**[7] ..... **C12P 21/02**
(52) **U.S. Cl.** ..... **435/69.1**; 435/358; 435/377
(58) **Field of Search** ..... 435/69.1, 377, 435/358

(56) **References Cited**

U.S. PATENT DOCUMENTS

| | | | | |
|---|---|---|---|---|
| 3,824,286 | A | | 7/1974 | Grimmelikhuysen et al. |
| 4,266,024 | A | | 5/1981 | Swetly et al. |
| 4,301,249 | A | * | 11/1981 | Markus et al. ..... 435/235.1 |
| 4,357,422 | A | | 11/1982 | Giard et al. |
| 4,416,986 | A | | 11/1983 | Markus et al. |
| 4,473,647 | A | | 9/1984 | Carpenter et al. |
| 4,992,472 | A | | 2/1991 | Driscoll et al. |
| 5,055,608 | A | | 10/1991 | Marks et al. |
| 5,330,744 | A | | 7/1994 | Pontremoli et al. |
| 5,674,834 | A | | 10/1997 | Theofan et al. |
| 5,705,364 | A | | 1/1998 | Etcheverry et al. |
| 5,712,163 | A | | 1/1998 | Parenteau et al. |
| 6,506,598 | B1 | | 1/2003 | Andersen et al. |
| 2001/0010922 | A1 | | 8/2001 | Dalla-Favera et al. |

FOREIGN PATENT DOCUMENTS

| | | |
|---|---|---|
| EP | 0 373 623 | 6/1990 |
| EP | 0 764 719 | 3/1997 |
| WO | WO 98/28010 | 7/1998 |
| WO | WO 99/32605 | 7/1999 |
| WO | WO 00/20576 | 4/2000 |

OTHER PUBLICATIONS

Beavo JA et al., "Effects of Xanthine derivatives on lipolysis and on adenosine 3', 5'–monophosphate phosphodiesterase activity, " *Mol Pharmacol* Nov. 1970; 6(6):597–603.

Carrasco L et al., "Antibiotics and compounds affecting translation by eukaryotic ribosomes, specific enhancement of aminoacyl–tRNA binding by methylxanthines," *Mol Cell Biochem* Feb. 1976; 10(2):97–122.

Cosgrove DE and Cox GS, "Enhancement by theophylline of the butyrate–mediated induction of choriogonadotropin α–subunit in HeLa cells," *Arch Biochem Biophys* Jul. 1990; 280(1):95–102.

Fernandez–Puentes C et al., "The enhancement of polypeptide synthesis in mammalian systems by methylxanthines," *FEBS Lett* Sep. 1974; 45(1):132–135.

Kaufmann H et al., "Comparative analysis of two controlled proliferation strategies regarding product quality, influence on tetracycline–regulated gene expression, and productivity," *Biotechnol Bioeng* 2001; 72(6):592–602.

Kaufmann H et al., "Influence of low temperature on productivity, proteome and protein phosphorylation of CHO cells," *Biotechnol Bioeng* 1999; 63:573–582.

Marks PA et al., "Inducing differentiation of transformed cells with hybrid polar compounds: a cell cycle–dependent process," *Proc Natl Acad Sci USA* Oct. 1994; 91:10251–10254.

Montague W and Cook JR, "Adenosine 3':5'–cyclic monophosphate and insulin release," *Biochem J* Dec. 1970; 120(4):9P–10P.

Montague W and Cook JR, "The role of adenosine 3':5'–cyclic monophosphate in the regulation of insulin release by isolated rat islets of langerhans," *Biochem J* 1971; 122:115–120.

Paterson T et al., "Approaches to maximizing stable expression of $\alpha_1$–antitrypsin in transformed CHO cells," *Appl Microbiol Biotechnol* 1994; 40:691–698.

Rajaraman R and Faulkner G, "Reverse transformation of chinese hamster ovary cells by methyl xanthines," *Exp Cell Res* 1984; 154:342–356.

Reuben RC et al., "A new group of potent inducers of differentiation in murine erythroleukemia cells," *Proc Natl Acad Sci USA* Mar. 1976; 73(3):862–866.

Richon VM et al., "A class of hybrid polar inducers of transformed cell differentiation inhibits histone deacetylases," *Proc Natl Acad Sci USA* Mar. 1998; 95:3003–3007.

Sarkaria JN et al., "Inhibition of ATM and ATR kinase activities by the radiosensitizing agent, caffeine," *Cancer Res* Sep. 1999; 59:4375–4382.

Schlegel R et al., "Exposure to caffeine and suppression of DNA replication combine to stabilize the proteins and RNA required for premature mitotic events," *J Cell Physiol* 1987; 131:85–91.

Staak K et al., "Pentoxifylline promotes replication of human cytomegalovirus in vivo and in vitro," *Blood* May 15, 1997; 89(10):3682–3690.

(List continued on next page.)

*Primary Examiner*—James Ketter
(74) *Attorney, Agent, or Firm*—Rosemary Sweeney

(57) **ABSTRACT**

The invention provides methods of increasing the production of polypeptides, optionally recombinant polypeptides, from mammalian cells using xanthine derivatives or hybrid polar compounds and cultures containing the same. Combinations of inducers including a hybrid polar compound and/or a xanthine derivative and/or an alkanoic acid can also be used, optionally at temperatures less than 37° C.

**69 Claims, 4 Drawing Sheets**

OTHER PUBLICATIONS

Tachibana H et al., "Induction of light chain replacement in human plasma cells by caffeine is independent from both the upregulation of RAG protein expression and germ line transcription," *J Biol Chem* Feb. 25, 2000; 275(8):5927–5933.

Takahashi K et al., "Growth rate suppression of cultured mammalian cells enhances protein productivity," *Cytotechnol* 1994; 15:57–64.

Tamura RN and Cox GS, "Enhancement by theophylline of the butyrate–mediated induction of choriogonadotropin α–subunit in HeLa cells," *Arch Biochem Biophys* Jul. 1990; 280(1):87–94.

Taylor WR and Stark GR, "Regulation of the G2/M transition by p53," *Oncogene* 2001; 20:1803–1815.

Zoumpourlis V and Spandidos DA, "Hexamethylene bisacetamide stimulates the expression of human immunodeficiency virus long terminal repeat sequences in rat and human fibroblasts," *Anti–Cancer Drugs* 1992; 3:163–167.

* cited by examiner

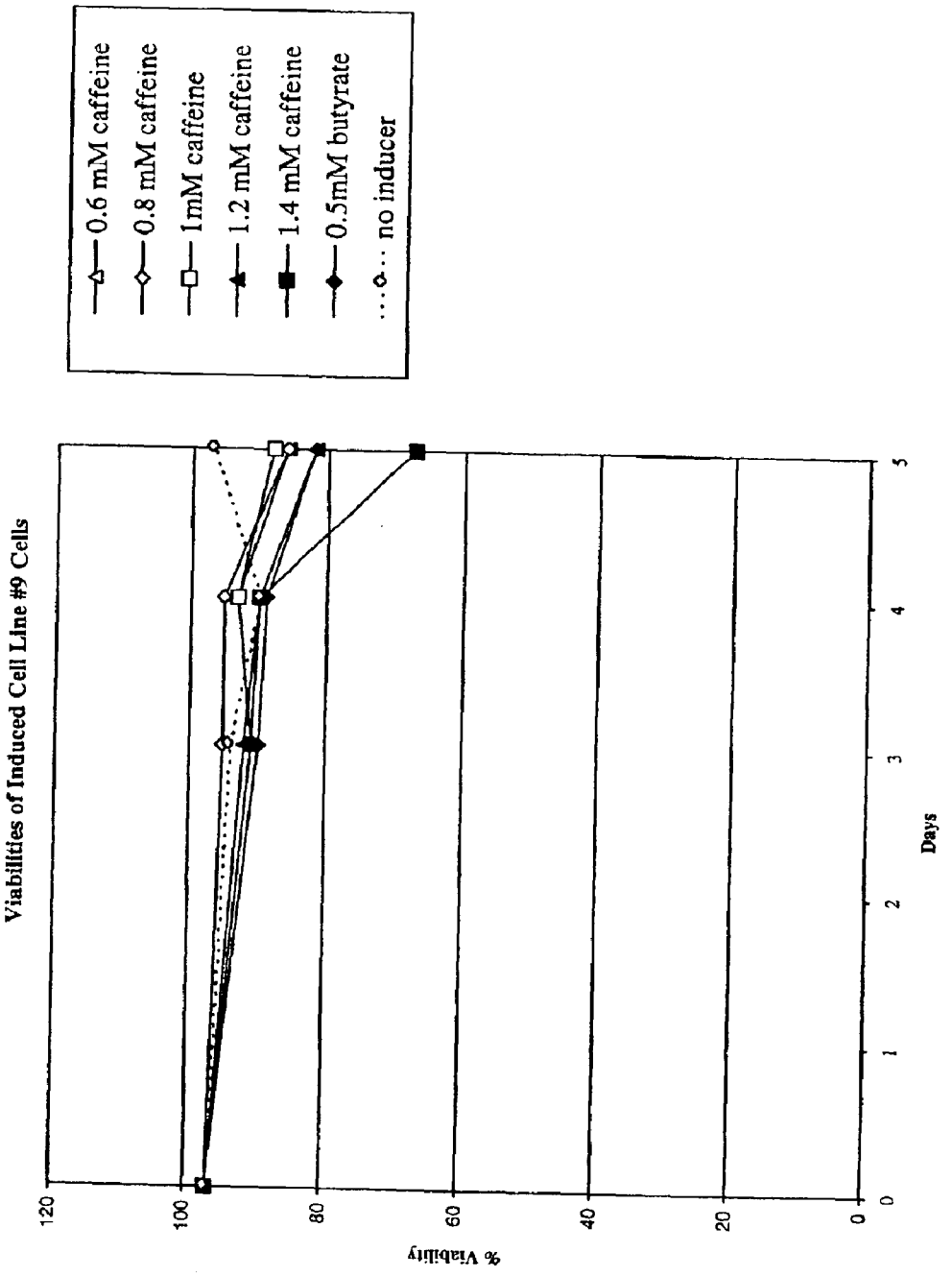

0.6 mM caffeine
0.8 mM caffeine
1mM caffeine
1.2 mM caffeine
1.4 mM caffeine
0.5mM butyrate
no inducer
Viabilities of Induced Cell Line #9 Cells
% Viability
120
100
80
60
40
20
0
Days
0
1
2
3
4
5

Figure 1

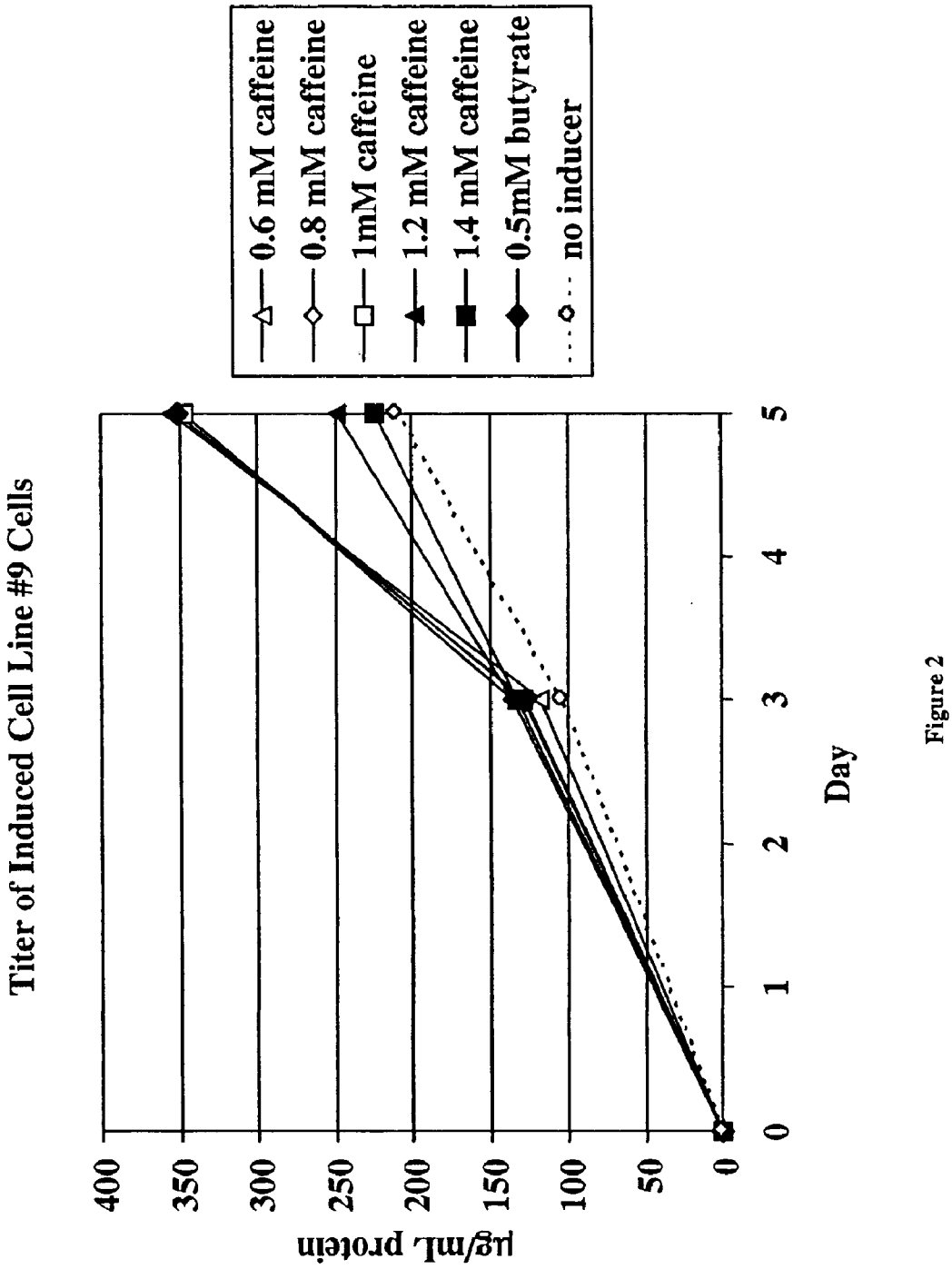

0.6 mM caffeine
0.8 mM caffeine
1mM caffeine
1.2 mM caffeine
1.4 mM caffeine
0.5mM butyrate
no inducer
Titer of Induced Cell Line #9 Cells
µg/mL protein
400
350
300
250
200
150
100
50
0
0
1
2
3
4
5
Day

Figure 2

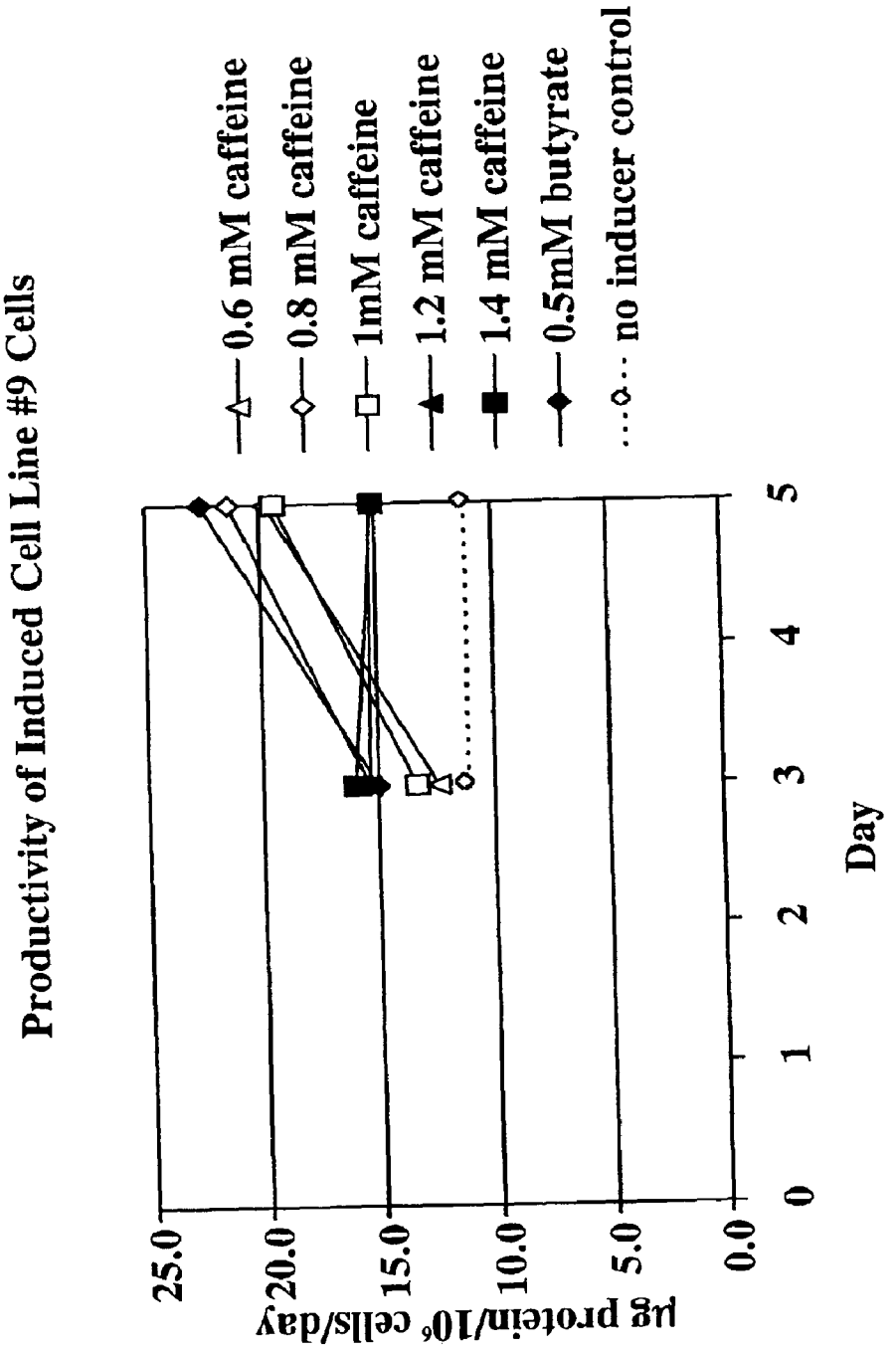

Productivity of Induced Cell Line #9 Cells
0.6 mM caffeine
0.8 mM caffeine
1mM caffeine
1.2 mM caffeine
1.4 mM caffeine
0.5mM butyrate
no inducer control
μg protein/10⁶ cells/day
25.0
20.0
15.0
10.0
5.0
0.0
0
1
2
3
4
5
Day

Figure 3

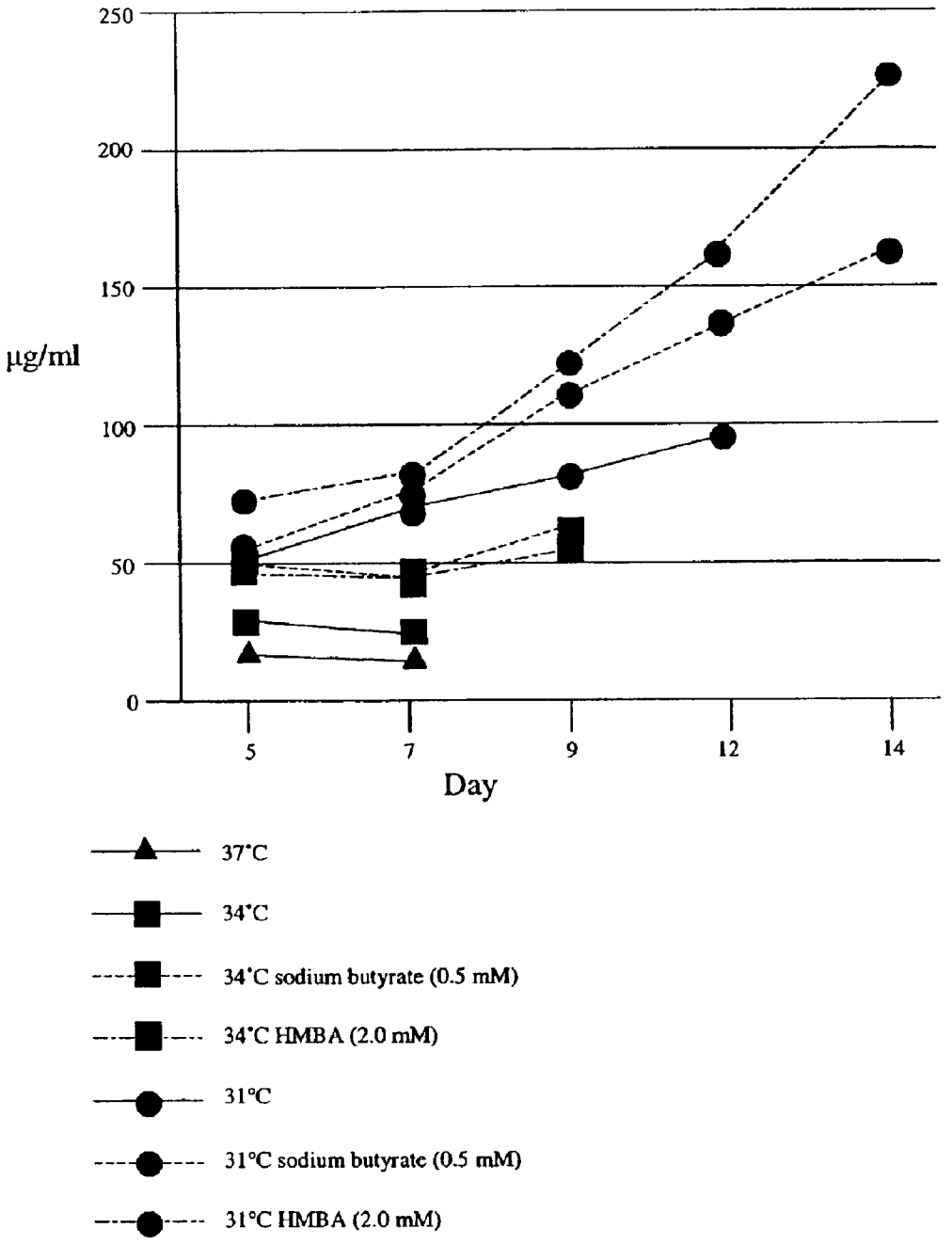

250
200
150
µg/ml
100
50
0
5
7
9
12
14
Day
37°C
34°C
34°C sodium butyrate (0.5 mM)
34°C HMBA (2.0 mM)
31°C
31°C sodium butyrate (0.5 mM)
31°C HMBA (2.0 mM)

Figure 4

# METHODS FOR INCREASING POLYPEPTIDE PRODUCTION

This application claims the benefit of U.S. Provisional Application Nos. 60/368,246 and 60/368,248, both filed Mar. 27, 2002, and both of which are incorporated herein in their entirety.

## FIELD OF THE INVENTION

The invention is in the field of polypeptide production, particularly recombinant polypeptide production in cell culture.

## BACKGROUND

Polypeptides are useful in a variety of diagnostic, therapeutic, agricultural, nutritional, and research applications. Although polypeptides can be isolated from natural sources, the isolation of large quantities of a specific polypeptide from natural sources may be expensive. Also, the polypeptide may not be of uniform quality due to variation in the source material. Recombinant DNA technology allows more uniform and cost-effective large-scale production of specific polypeptides.

One goal of recombinant polypeptide production is the optimization of culture conditions so as to obtain the greatest possible productivity. Incremental increases in productivity can be economically significant. Some of the methods to increase productivity in cell culture include using enriched medium, monitoring osmolarity during production, decreasing temperatures during specific phases of a cell culture, and/or the addition of sodium butyrate (see, e.g., U.S. Pat. No. 5,705,364).

However, as more polypeptide-based drugs demonstrate clinical effectiveness and increased commercial quantities are needed, available culture facilities become limited. Accordingly, there remains a need in the art to continually improve yields of recombinant polypeptides from each cell culture run.

## SUMMARY

As shown by the experimental data reported herein, xanthine derivatives and/or hybrid polar compounds can dramatically induce the production of polypeptides, especially recombinant polypeptides, from mammalian cell lines. Moreover, xanthine derivatives and/or hybrid polar compounds can be used in combination with other induction methods to further increase polypeptide expression.

Thus, in one aspect, the invention provides a method for producing a polypeptide, which may be a recombinant polypeptide, comprising culturing a mammalian cell line in a growth phase followed by a production or induction phase, which can occur at a temperature of less than 37° C., and adding to the culture during the production phase a xanthine derivative. The addition of the xanthine derivative can increase the production of the polypeptide. The mammalian cell line can be a cell line that has been genetically engineered to produce the polypeptide or a hybridoma cell line that can produce an antibody. The xanthine derivative may be caffeine at a concentration from about 0.01 millimolar to about 5.0 millimolar or from about 0.01 millimolar to about 3.0 millimolar. In some embodiments, the mammalian cell line is a CHO cell line, and it may have been transformed with a recombinant vector encoding the recombinant polypeptide. Optionally, the vector can comprise a cytomegalovirus (CMV) promoter. Typically, the cell does not naturally express the polypeptide or only naturally expresses the polypeptide at very low levels (in the absence of genetic engineering). The polypeptide may be a recombinant fusion polypeptide or a human or humanized antibody. The production or induction phase can occur at a temperature from about 29° C. to about 36° C. or from about 30° C. to about 33° C. The growth phase can occur at a temperature from about 35° C. to about 38° C.

Optionally, at least two different xanthine derivatives can be added. The xanthine derivative(s) can be selected from the group consisting of caffeine, 3-isobutyl-1-methylxanthine, theophylline, theobromine, pentoxyphylline, and aminophylline or from a subset of this group. If two different xanthine derivatives are added, they can be caffeine and 3-isobutyl-1-methylxanthine. Xanthine derivatives can be added multiple times during the culturing of the cell line, and the cell line can be cultured in the presence of the xanthine derivative for at least about 5 days. The concentration of each xanthine derivative added to the culture can be from about 0.001 millimolar to about 3 millimolar. The recombinant polypeptide can be collected from the medium and formulated. The medium may further comprise a hybrid polar compound and/or an alkanoic acid. The hybrid polar compound can be hexamethylene bisacetamide, optionally at a concentration from about 0.1 millimolar to about 5 millimolar. The xanthine derivative can be caffeine, optionally at a concentration from about 0.1 millimolar to about 4 millimolar. The alkanoic acid can be a salt of butyric acid, optionally at a concentration from about 0.1 millimolar to about 2 millimolar. The mammalian cells can be cultured at a temperature from about 29° C. to about 36° C. or from about 30° C. to about 33° C. The mammalian cells can be cultured in a growth phase at a first temperature from about 35° C. to about 38° C. before they are shifted to a production phase at a second temperature from about 29° C. to about 36° C., wherein the second temperature can be lower than the first temperature. The xanthine can be added at the time of the shift from the first temperature to the second temperature and/or before and/or after the shift.

In another aspect the invention provides a method for producing a recombinant polypeptide comprising growing in culture a mammalian cell line, optionally a CHO cell line that has been genetically engineered to produce the recombinant polypeptide, and adding to the culture medium at least one xanthine derivative selected from the group consisting of theobromine and caffeine. The addition of the xanthine derivative can increase the production of the recombinant polypeptide. The mammalian cell line may have been transformed with a recombinant vector encoding the recombinant polypeptide. Optionally, the vector can comprise a cytomegalovirus (CMV) promoter. Typically, the cell line does not naturally express the recombinant polypeptide or only naturally expresses the recombinant polypeptide at very low levels (in the absence of genetic engineering). The recombinant polypeptide may be a recombinant fusion polypeptide or a human or humanized antibody. The cell line can be cultured in a growth phase, which is distinct from a production or induction phase. The production phase can occur at a temperature less than 37° C. The cell line can be cultured at a temperature of from about 29° C. to about 36° C. or from about 30° C. to about 33° C. Optionally, at least two different xanthine derivatives can be added. Xanthine derivatives can be added multiple times during the culturing of the cell line. The concentration of each xanthine derivative added to the culture can be from about 0.001 millimolar to about 3 millimolar. The recombinant polypeptide can be

collected from the medium and formulated. The mammalian cell line can be cultured at a first temperature from about 35° C. to about 38° C. before it is shifted to a second temperature from about 29° C. to about 36° C., and the xanthine derivative can be added at the time of the shift from the first temperature to the second temperature and/or before and/or after the shift. The second temperature can be lower than the first temperature.

In another aspect, the invention provides a culture comprising a CHO cell genetically engineered to produce a polypeptide, a production medium, and at least one xanthine derivative selected from the group consisting of caffeine, 3-isobutyl-1-methylxanthine, theophylline, theobromine, pentoxyphylline, and aminophylline or from a subset of this group. The culture can comprise at least two xanthine derivatives. The concentration of each xanthine derivative present can be from about 0.001 millimolar to about 3 millimolar or from about 0.01 millimolar to about 3 millimolar. The culture can comprise serum-free medium, and may comprise no added protein or may comprise insulin or IGF-1. Additionally, the culture can comprise dimethylformamide, dimethylsulfoxide, or dimethylacetamide. The invention, because of its low cost and convenience, is particularly useful for large scale culturing of CHO cells. The culture can be a large scale culture of at least 100 liters, or even at least 500 liters, in size. The culture can comprise a homogeneous CHO cell line.

In still another aspect, the invention encompasses a culture comprising a CHO cell genetically engineered to produce a polypeptide, a production medium, and at least one xanthine derivative, wherein the culture is grown at less than 37° C. for at least part of its life. The xanthine derivative or derivatives present can be within the concentration range from about 0.001 millimolar to about 3 millimolar or from about 0.01 millimolar to about 3 millimolar, and the culture can contain at least two different xanthine derivatives. The xanthine derivatives can be selected from the group consisting of caffeine, 3-isobutyl-1-methylxanthine, theophylline, theobromine, pentoxyphylline, and aminophylline or from a subset of this group such as caffeine, theobromine and pentoxyphylline. The size of the culture can be at least 100 liters, and the production medium can be serum-free medium and can comprise either no added protein or insulin or IGF-1. The culture can comprise a homogeneous CHO cell line.

In a further aspect, the invention includes a method for producing a polypeptide in a culture of mammalian cells comprising incubating the culture at a temperature of about 37° C. and thereafter incubating the culture at a temperature from about 29° C. to 36° C., and adding to the culture a xanthine derivative during the incubation at a temperature from about 29° C. to 36° C., wherein the polypeptide is a recombinant polypeptide or an antibody. The xanthine derivative can be selected from the group consisting of caffeine, 3-isobutyl-1-methylxanthine, theophylline, theobromine, pentoxyphylline, and aminophylline or from a subset of this group such as caffeine, theobromine, and pentoxyphylline. The mammalian cells can be hybridoma cells or CHO cells. The xanthine derivative or derivatives present can be within the concentration range from about 0.001 millimolar to about 3 millimolar or from about 0.01 millimolar to about 3 millimolar, and the culture can contain at least two different xanthine derivatives. Xanthine derivatives can be added multiple times during the culturing of the cell line.

The invention provides a method for producing a recombinant polypeptide comprising culturing a mammalian cell line, in some embodiments a CHO cell line, at a temperature from about 29° C. to about 36° C., optionally at temperatures between about 29° C. and 35° C. or from about 30° C. to about 33° C., in a medium comprising a hybrid polar compound. The medium can be serum free. The addition of the hybrid polar compound can increase the production of the recombinant polypeptide. The hybrid polar compound can be hexamethylene bisacetamide, optionally at a concentration from about 0.1 millimolar to about 20 millimolar or from about 0.1 millimolar to about 5 millimolar. Furthermore, the medium may comprise an alkanoic acid, such as a salt of butyric acid, at a concentration, for example, from about 0.05 millimolar to about 10 millimolar, optionally from about 0.1 millimolar to about 2 millimolar. Furthermore, the medium may comprise a xanthine derivative, for example, caffeine, at a concentration from about 0.005 millimolar to 10 millimolar, optionally from about 0.01 millimolar to 4 millimolar or from about 0.1 millimolar to 4 millimolar. The mammalian cells can be cultured at a first temperature from about 35° C. to about 38° C. before they are shifted to a second temperature between about 29° C. and 36° C., and the hybrid polar compound can be added after the shift from the first temperature to the second temperature. The mammalian cells may be genetically engineered to produce a polypeptide, optionally a secreted polypeptide that can be recovered from the medium, including RANK:Fc, type II interleukin-1 receptor, TNFR:Fc, CD40 ligand, TRAIL, flt3-ligand, IL-4 receptor, G-CSF, erythropoietin, an antibody, or a substantially similar polypeptide, among others.

In another embodiment, the invention provides an improved method for producing a polypeptide by culturing mammalian cells comprising culturing the cells in a medium comprising a hybrid polar compound, optionally at temperatures from about 29° C. to about 36° C., between about 29° C. and 35° C., or from about 30° C. to about 33° C. The hybrid polar compound may be hexamethylene bisacetamide, optionally at a concentration between about 0.1 millimolar and about 5 millimolar. The addition of the hybrid polar compound can increase the production of the polypeptide, which may be a recombinant polypeptide. Furthermore, the medium may comprise an alkanoic acid, for example, butyric acid, optionally at a concentration from about 0.05 millimolar to about 10 millimolar or from about 0.1 millimolar to about 2 millimolar. Furthermore, the medium may comprise a xanthine, such as, for example, caffeine, optionally at a concentration from about 0.005 millimolar to 10 millimolar or from about 0.01 millimolar to 5 millimolar. Optionally, the polypeptide may be RANK:Fc, type II interleukin-1 receptor, TNFR:Fc, CD40 ligand, TRAIL, flt3-ligand, IL-4 receptor, GM-CSF, erythropoietin, an antibody, or a substantially similar polypeptide among others.

In another aspect, the invention provides a method for obtaining a polypeptide, optionally a recombinant polypeptide, comprising recovering the polypeptide from medium in which mammalian cells have been grown, wherein the mammalian cells can secrete the polypeptide and are grown at temperatures between about 29° C. and 35° C., optionally from about from about 30° C. to about 33° C., in medium comprising hexamethylene bisacetamide. The hexamethylene bisacetamide may be present at concentrations between about 0.1 millimolar and about 5 millimolar. Furthermore, the medium may comprise an alkanoic acid, for example, butyric acid, optionally at a concentration from about 0.05 millimolar to about 10 millimolar or from about 0.1 millimolar to about 2 millimolar. Furthermore, the

medium may comprise a xanthine, for example, caffeine, optionally at a concentration from about 0.005 millimolar to 10 millimolar or from about about 0.01 millimolar to 5 millimolar. The polypeptide may be RANK:Fc, type II interleukin-1 receptor, TNFR:Fc, CD40 ligand, TRAIL, flt3-ligand, IL-4 receptor, G-CSF, erythropoietin, an antibody, or a substantially similar polypeptide, among others.

In a further embodiment, the invention comprises method for producing a recombinant polypeptide comprising culturing mammalian cells in a medium comprising a hybrid polar compound and a xanthine, wherein the mammalian cells have been genetically engineered to express the recombinant polypeptide. The medium may further comprise an alkanoic acid, such as, for example, a salt of butyric acid, which may be at a concentration from about 0.1 millimolar to about 2 millimolar. The hybrid polar compound can be hexamethylene bisacetamide, which may be at a concentration from about 0.1 millimolar to about 5 millimolar, and/or the xanthine can be caffeine, which may be at a concentration from about 0.1 millimolar to about 4 millimolar. The cells can be cultured at a temperature from about 29° C. to about 36° C. or from about 30° C. to about 33° C. The mammalian cells can be cultured at a first temperature from about 35° C. to about 38° C. before they are shifted to a second temperature from about 29° C. to about 36° C., and the hybrid polar compound and the xanthine can be added at the time of the shift from the first temperature to the second temperature and/or before and/or after the shift. The can be medium can be serum free.

In a further embodiment, the invention encompasses a method for producing a polypeptide, optionally a recombinant polypeptide, comprising culturing mammalian cells in a medium comprising a hybrid polar compound and an alkanoic acid, wherein the mammalian cells may have been genetically engineered to express the recombinant polypeptide. The hybrid polar compound can be hexamethylene bisacetamide, and the hybrid polar compound can be present at a concentration of from about 0.5 millimolar to about 10 millimolar or at a concentration between about 0.5 millimolar and 2.5 millimolar. The alkanoic acid can be a salt of butyric acid, and the alkanoic acid can be present at a concentration from about 0.1 millimolar to about 5 millimolar or at a concentration between about 0.1 millimolar and about 2.0 millimolar. The mammalian cells can be cultured at a temperature from about 29° C. to about 36° C., and the medium can be serum free. The mammalian cell line can be cultured at a first temperature from about 35° C. to about 38° C. before they are shifted to a second temperature from about 29° C. to about 36° C., and the hybrid polar compound and the alkanoic acid may be added after the shift from the first temperature to the second temperature. The medium can further comprise a xanthine derivative at a concentration from about 0.001 millimolar to about 5.0 millimolar. The mammalian cell line can be a hybridoma cell line or a CHO cell line.

In still another embodiment, the invention provides a method for producing a polypeptide comprising culturing a mammalian cell line in a production phase at a second temperature from about 30° C. to 34° C. in a medium comprising a hybrid polar compound, wherein the production phase follows a growth phase at a first temperature from about 35° C. to about 38° C. The polypeptide can be a recombinant polypeptide or an antibody. The hybrid polar compound can be hexamethylene bisacetamide, optionally at a concentration from about 0.1 millimolar to about 5 millimolar. The hybrid polar compound may be added after the shift from the first temperature to the second temperature. The medium can further comprise an alkanoic acid, which can be a salt of butyric acid, optionally at a concentration from about 0.05 millimolar to about 10.0 millimolar. The medium can also comprise a xanthine derivative, optionally at a concentration from about 0.001 millimolar to about 5.0 millimolar. The medium can be serum free. The mammalian cell line can be a hybridoma cell line or a CHO cell line.

The invention also provides a method for producing a polypeptide comprising culturing a mammalian cell line in a medium comprising a hybrid polar compound at a concentration between about 0.5 millimolar and 2.5 millimolar, an alkanoic acid at a concentration from about 0.1 millimolar and 2.0 millimolar, and a xanthine derivative at a concentration from about 0.001 millimolar to about 4 millimolar.

In still another embodiment, the invention provides a method for producing a polypeptide, optionally RANK:Fc, type II interleukin-1 receptor, TNFR:Fc, CD40 ligand, TRAIL, flt3-ligand, IL-4 receptor, G-CSF, erythropoietin, an antibody, or a substantially similar polypeptide, comprising culturing mammalian cells, which may have been genetically engineered to produce any of these polypeptides, in a medium comprising between about 0.1 millimolar and about 5 millimolar HMBA, from about 0.1 millimolar to about 2 millimolar butyric acid, and from about 0.1 millimolar to about 4 millimolar caffeine at a temperature from about 29° C. to about 36° C. or from about 30° C. to about 33° C.

## BRIEF DESCRIPTION OF THE FIGURES

FIG. **1** shows the percentage of the total cells that are viable under the indicated conditions at 31° C. for the #9 CHO cell line as a function of days in culture.

FIG. **2** shows the micrograms of protein per milliliter of cell culture, i.e., protein titer, under the indicated conditions at 31° C. for the #9 CHO cell line as a function of days in culture.

FIG. **3** shows the micrograms of protein per $10^6$ cells per day under the indicated conditions at 31° C. for #9 CHO cell line as a function of days in culture.

FIG. **4** shows a graph displaying the concentration of an antibody against murine IL-4 receptor recovered from medium as a function of days of growth of a CHO cell line comprising a vector encoding the antibody at the stated temperatures in the presence or absence of HMBA or sodium butyrate. Markings are as follows: —▲—, no inducer 37° C.; —■—, no inducer 34° C.; - -■- - -, 0.5 millimolar sodium butyrate 34° C.; - - -■- -, 2.0 millimolar HMBA 34° C.; —●—, no inducer 31° C.; - - -●- - -, 0.5 millimolar sodium butyrate 31° C.; and - - -● - - -, 2.0 millimolar HMBA 31° C.

## DETAILED DESCRIPTION OF THE INVENTION

An "antibody" is a polypeptide or complex of polypeptides, each of which comprises at least one variable antibody immunoglobulin domain and at least one constant antibody immunoglobulin domain. Antibodies may be single chain antibodies, dimeric antibodies, or some higher order complex of polypeptides including, but not limited to, heterodimeric antibodies. A "human antibody" is an antibody encoded by nucleic acids that are ultimately human in origin. Such an antibody can be expressed in a non-human cell or organism. For example, DNA encoding a human

antibody can be introduced into tissue culture cells and expressed in transformed cell lines. Alternatively, human antibodies can be expressed in transgenic animals such as, for example, the transgenic mice described in Mendez et al. ((1997), Nature Genetics 16(4): 146–56). Such transgenic mice are utilized in making the fully human antibodies in U.S. Pat. No. 6,235,883 B1. Human antibodies can also be expressed in hybridoma cells. A "humanized antibody" is a chimeric antibody comprising complementarity determining regions (CDR1, CDR2, and CDR3) from a non-human source and other regions that conform to sequences in human antibodies (and may be of human origin) as explained in, e.g., U.S. Pat. Nos. 5,558,864 and 5,693,761 and International Patent Application WO 92/11018.

A "constant antibody immunoglobulin domain" is an immunoglobulin domain that is identical to or substantially similar to a $C_L$, $C_H1$, $C_H2$, $C_H3$, or $C_H4$, domain of human or animal origin. See e.g. Hasemann and Capra, Immunoglobulins: Structure and Function, in William E. Paul, ed., Fundamental Immunology, Second Edition, 209, 210–218 (1989); Kabat et al., Sequences of Proteins of Immunological Interest, U.S. Dept. of Health and Human Services (1991).

An "$F_C$ portion of an antibody" includes human or animal immunoglobulin domains $C_H2$ and $C_H3$ or immunoglobulin domains substantially similar to these. For discussion, see Hasemann and Capra, supra, at 212–213 and Kabat et al., supra.

Cells have been "genetically engineered" to express a specific polypeptide when recombinant nucleic acid sequences that allow expression of the polypeptide have been introduced into the cells using methods of "genetic engineering," such as viral infection with a recombinant virus, transfection, transformation, or electroporation. See e.g. Kaufman et al. (1990), Meth. Enzymol. 185: 487–511; *Current Protocols in Molecular Biology*, Ausubel et al., eds. (Wiley & Sons, New York, 1988, and quarterly updates). Infection with an unaltered, naturally-occurring virus, such as, for example, hepatitis B virus, human immunodeficiency virus, adenovirus, etc., does not constitute genetic engineering as meant herein. The term "genetic engineering" refers to a recombinant DNA or RNA method used to create a host cell that expresses a gene at elevated levels or at lowered levels, or expresses a mutant form of the gene. In other words, the cell has been transfected, transformed or transduced with a recombinant polynucleotide molecule, and thereby altered so as to cause the cell to alter expression of a desired polypeptide. For the purposes of the invention, the antibodies produced by a hybridoma cell line resulting from a cell fusion are not "recombinant polypeptides." Further, viral polypeptides produced by a cell as a result of viral infection are also not "recombinant polypeptides" as meant herein unless the viral nucleic acid has been altered by genetic engineering prior to infecting the cell. The methods of "genetic engineering" also encompass numerous methods including, but not limited to, amplifying nucleic acids using polymerase chain reaction, assembling recombinant DNA molecules by cloning them in *Escherichia coli*, restriction enzyme digestion of nucleic acids, ligation of nucleic acids, and transfer of bases to the ends of nucleic acids, among numerous other methods that are well-known in the art. See e.g. Sambrook et al., Molecular Cloning: A Laboratory Manual, 2nd ed., vol. 1–3, Cold Spring Harbor Laboratory, 1989. Methods and vectors for genetically engineering cells and/or cell lines to express a polypeptide of interest are well known to those skilled in the art. Genetic engineering techniques include but are not limited to expression vectors, targeted homologous recombination and gene activation (see, for example, U.S. Pat. No. 5,272,071 to Chappel) and trans activation by engineered transcription factors (see e.g., Segal et al., 1999, Proc. Natl. Acad. Sci. USA 96(6) :2758–63). Optionally, the polypeptides are expressed under the control of a heterologous control element such as, for example, a promoter that does not in nature direct the production of that polypeptide. For example, the promoter can be a strong viral promoter (e.g., CMV, SV40) that directs the expression of a mammalian polypeptide. The host cell may or may not normally produce the polypeptide. For example, the host cell can be a CHO cell that has been genetically engineered to produce a human polypeptide, meaning that nucleic acid encoding the human polypeptide has been introduced into the CHO cell. Alternatively, the host cell can be a human cell that has been genetically engineered to produce increased levels of a human polypeptide normally present only at very low levels (e.g., by replacing the endogenous promoter with a strong viral promoter).

"Growth phase" means a period during which cultured cells are rapidly dividing and increasing in number. During growth phase, cells are generally cultured in a medium and under conditions designed to maximize cell proliferation.

A "hybrid polar compound" is compound having two polar groups separated by an apolar carbon chain. This includes hexamethylene bisacetamide (HMBA) and the other molecules discussed below and in the following references: Richon et al. (1998), Proc. Natl. Acad. Sci. 95: 3003–07; Marks et al. (1994), Proc. Natl. Acad. Sci. 91: 10251–54; and U.S. Pat. Nos. 5,055,608 and 6,087,367.

The production of a polypeptide is "increased" by the addition of an inducing agent, such as hexamethylene bisacetamide (HMBA) or caffeine, if the amount the polypeptide produced in a culture containing the inducing agent is more than the amount of the polypeptide produced in an otherwise identical culture that does not contain the inducing agent. Similarly, the production of a polypeptide is "increased" by growth at a temperature other than 37° C. if the amount of polypeptide produced in a culture incubated at a temperature other than 37° C. is more than the amount of the polypeptide produced in an otherwise identical culture incubated at 37° C.

A "multimerization domain" is a domain within a polypeptide molecule that confers upon it a propensity to associate with other polypeptide molecules through covalent or non-covalent interactions.

A "naturally-occurring polypeptide" is a polypeptide that occurs in nature, that is, a polypeptide that can be produced by cells that have not been genetically engineered. Such a polypeptide may also be produced by cells genetically engineered to produce it.

"Polypeptide" means a chain of at least 6 amino acids linked by peptide bonds. Optionally, a polypeptide can comprise at least 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 150, 200, 250, or 300 amino acids linked by peptide bonds.

"Production medium" means a cell culture medium designed to be used to culture cells during a production phase.

"Production phase" means a period during which cells are producing maximal amounts of recombinant polypeptide. A production phase is characterized by less cell division than during a growth phase and by the use of medium and culture conditions designed to maximize polypeptide production.

A "recombinant fusion polypeptide" is a fusion of all or part of at least two polypeptides, which is made using the methods of genetic engineering.

A "recombinant polypeptide" is a polypeptide resulting from the process of genetic engineering. For the purposes of the invention, the antibodies produced by a hybridoma cell line resulting from a cell fusion are not "recombinant polypeptides." Further, viral proteins produced by a cell as a result of viral infection with a naturally-occurring virus are also not "recombinant polypeptides" as meant herein unless the viral nucleic acid has been altered by genetic engineering prior to infecting the cell.

"Substantially similar" polypeptides are at least 80%, optionally at least 90%, identical to each other in amino acid sequence and maintain or alter in a desirable manner the biological activity of the unaltered polypeptide. Conservative amino acid substitutions, unlikely to affect biological activity, include, without limitation, the following: Ala for Ser, Val for Ile, Asp for Glu, Thr for Ser, Ala for Gly, Ala for Thr, Ser for Asn, Ala for Val, Ser for Gly, Tyr for Phe, Ala for Pro, Lys for Arg, Asp for Asn, Leu for Ile, Leu for Val, Ala for Glu, Asp for Gly, and these changes in the reverse. See e.g. Neurath et al., *The Proteins*, Academic Press, New York (1979). In addition exchanges of amino acids among members of the following six groups of amino acids will be considered to be conservative substitutions for the purposes of the invention. The groups are: 1) methionine, alanine, valine, leucine, and isoleucine; 2) cysteine, serine, threonine, asparagine, and glulamine; 3) aspartate and glutamate; 4) histidine, lysine, and arginine; 5) glycine and proline; and 6) tryptophan, tyrosine, and phenylalanine. The percent identity of two amino sequences can be determined by visual inspection and mathematical calculation, or more preferably, the comparison is done by comparing sequence information using a computer program such as the Genetics Computer Group (GCG; Madison, Wis.) Wisconsin package version 10.0 program, 'GAP' (Devereux et al. (1984), *Nucl. Acids Res*. 12: 387) or other comparable computer programs. The preferred default parameters for the 'GAP' program includes: (1) the weighted amino acid comparison matrix of Gribskov and Burgess (1986), *Nucl. Acids Res*. 14: 6745, as described by Schwartz and Dayhoff, eds., *Atlas of Polypeptide Sequence and Structure*, National Biomedical Research Foundation, pp. 353–358 (1979), or other comparable comparison matrices; (2) a penalty of 30 for each gap and an additional penalty of 1 for each symbol in each gap for amino acid sequences; (3) no penalty for end gaps; and (4) no maximum penalty for long gaps. Other programs used by those skilled in the art of sequence comparison can also be used.

"Transition phase" means a period of cell culture between a "growth phase" and a "production phase." During transition phase, the medium and environmental conditions are typically shifted from those designed to maximize proliferation to those designed to maximize polypeptide production.

A "variable antibody immunoglobulin domain" is an immunoglobulin domain that is identical or substantially similar to a $V_L$ or a $V_H$ domain of human or animal origin.

The present invention is directed towards improved methods for culturing mammalian cells, which may have been genetically engineered to produce a particular polypeptide. In particular, the invention is directed towards culture methods that maximize the production of specific polypeptides. It is also directed towards methods of producing and obtaining such polypeptides from cultured mammalian cells. Polypeptides are useful in a large variety of diagnostic, therapeutic, agricultural, nutritional, and research applications.

As shown by the experimental data reported herein, it has been discovered that xanthine derivatives and hybrid polar compounds used separately or together can dramatically induce the production of recombinant polypeptide from CHO cell lines. In particular, addition of the xanthine derivative caffeine to the production phase of a cell culture enhances recombinant polypeptide production. The hybrid polar compound hexamethylene bisacetamide is also shown to be an effective inducer of recombinant polypeptide production. Further, other inducers, such as, for example, alkanoic acids, can also be added to either a xanthine derivative, a hybrid polar compound, or both. Other methods, such as, for example, culturing the cells at temperatures from about 29° C. to about 36° C., between about 29° C. and 35° C., and/or from about 30° C. to about 33° C. can also be used. Thus, the invention relates to inducing increased production of a recombinant polypeptide from a cell grown in culture by exposing the cell to chemical inducers, including hybrid polar compounds and/or xanthine derivatives.

The methods of the invention include culturing mammalian cells in medium comprising a hybrid polar compound, for example, hexamethylene bisacetamide (HMBA), optionally at temperatures between about 29° C. and 35° C. or from about 30° C. to about 33° C. Other embodiments of the invention encompass culture conditions in which an alkanoic acid and/or a xanthine, in addition to the hybrid polar compound, are added to the culture medium. In one embodiment, a xanthine and a hybrid polar compound and culture temperatures between about 29° C. and 36° C. are used. Another embodiment comprises the addition of an alkanoic acid and a hybrid polar compound plus culture temperatures between about 29° C. and 36° C. Still another embodiment comprises addition of a xanthine, an alkanoic acid, and a hybrid polar compound plus culture temperatures between about 29° C. and 36° C. Optionally, cell culture using the methods of the invention can take place during a production phase, as distinguished from a growth phase. A growth phase can be distinguished from a production phase by, for example, a temperature shift and/or a change in medium such as, for example, the addition of one or more inducers.

In one aspect, the invention provides a method comprising growing in culture a mammalian cell that has been genetically engineered to produce a polypeptide; and adding to the culture a xanthine derivative. A genetically engineered cell may be a cell that has been transformed with a recombinant vector encoding the polypeptide. In addition, the polypeptide can be expressed under the control of a heterologous promoter such as, for example, a CMV promoter. Typically, the cell does not naturally express the polypeptide or only naturally expresses the polypeptide at very low levels (in the absence of genetic engineering). In another aspect, the invention provides a culture containing a cell genetically engineered to produce a polypeptide, a production medium, and the xanthine derivative.

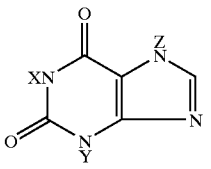
In addition, the methods and compositions of the invention can be used in combination with any other known or yet to be discovered methods of inducing the production of recombinant polypeptides. Such techniques include cold temperature shift, alkanoic acid additions (as described in U.S. Pat. No. 5,705,364 to Etcheverry et al., incorporated herein by reference), DMF, and DMSO, to name just a few examples, as well as any yet to be described and/or discovered induction techniques. As used herein, "inducing" polypeptide production or "induction" refers to culturing cells under a set of conditions designed to maximize the total amount of a desired polypeptide made by the cells. An "inducer" is an agent that, when added to culture medium,

can increase the production of a desired polypeptide in at least some cell lines. Combining the addition of xanthine derivatives with other protein induction techniques can have a synergistic effect on polypeptide induction, allowing for lower additions of xanthine derivatives and/or lower additions of other inducing agents and/or more conservative temperature shifts. The other methods of induction can take place at around the same time as xanthine addition, and/or before and/or after xanthine addition. For example, one can shift the temperature of the culture at day 0, and then add a xanthine derivative and/or a hybrid polar compound, and optionally other chemical inducers, later, e.g. one to several hours or days later. Such a protocol allows some additional growth of a seeded culture before full induction. Furthermore, multiple additions of xanthine derivatives and/or hybrid polar compounds can be added to the culture during the production phase, separated by about 12, 24, 48, and/or 72 hours or more, with or without additions of other inducing agents or changes in culture conditions. For example, an inducer can be added at day 0 and again at day 4. Alternatively, an inducer can be added for the first time one, two, three, or four days after a temperature shift.

In one aspect, the invention entails performing a low temperature shift (shifting the temperature of the medium from the optimal growth temperature, usually around 37° C., to a lower temperature, usually from about 29° C. to about 36° C., and optionally about 30° C. to about 34° C. at the time of, before, and/or after adding the xanthine derivative or the hybrid polar compound. Alternatively, or in addition, an alkanoic acid or salt thereof (e.g. sodium butyrate) can be added to the culture at around the same time as the xanthine derivative and/or hybrid polar compound is added. Alkanoic acid can be added at concentrations typically used for induction, or even at lower concentrations than would typically be used. Thus, by manipulating both transcriptional and post-transcriptional controls, higher levels of productivity may be achieved.

There are individual differences between cell lines in the effectiveness of various inducers. For example, although sodium butyrate is a widely-used inducer, it can have no effect or an adverse effect on polypeptide production in some cell lines. See Table 5. Different inducers or different concentrations of the same inducers may be appropriate for different cell lines. Furthermore, different temperatures may be appropriate for different cell lines. In spite of this variability, some inducers, such as, for example, caffeine, hexamethylene bisacetamide, and sodium butyrate, can be useful in a wide variety, though perhaps not all, cell lines.

Generally, xanthine derivatives have the structure illustrated below.




X, Y, and Z can be independently selected from a straight or branched chain alkyl radical having from 1 to 12 carbons, a straight or branched chain alkynyl radical having from 1 to 12 carbons (including a propynyl radical), a straight or branched chain acyl radical having from 1 to 12 carbons, a straight or branched chain radical with the structure —R-acyl containing from 1 to 12 carbons where R is a saturated or unsaturated aliphatic group, a straight or branched chain allenyl radical having from 1 to 12 carbons, a straight or branched chain hydroxyalkyl radical having from 1 to 12 carbons, a straight or branched chain hydroxyallenyl radical having from 1 to 12 carbons, a straight or branched chain radical with the structure -allenyl-halogen having from 1 to 12 carbons, a cyclohexyl radical, and hydrogen. In some embodiments, at least one of X, Y and Z is a methyl group. In some embodiments, each of X and Y independently represents a hydrogen atom, a linear or branched alkyl radical having up to 5 carbon atoms, an allyl radical, a propynyl radical or a cyclohexyl radical, with the proviso that X and Y do not simultaneously represent a hydrogen atom, and Z represents a hydrogen, methyl, ethyl, hydroxymethyl, hydroxyethyl or heterocyclo radical. These xanthines can be obtained using conventional processes and/or purchased. A number of different xanthine derivatives that can be used are described in Beavo et al. (1970), Molec. Pharm. 6:597–603, and incorporated by reference herein.

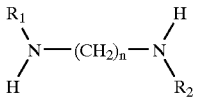
Illustrative examples of xanthine derivatives that can be used in the methods and compositions of the invention include, but are not limited to, caffeine (1,3,7-trimethylxanthine), theophylline (1,3-dimethylxanthine), theobromine (3,7-dimethylxanthine), 3-isobutyl-1-methylxanthine, 3-butyl-1-methylxanthine, 1,3,7-triethylxanthine, 3-cyclohexyl-1-ethylxanthine, 3-ethyl-1-propynylxanthine, 3-ethyl-1-pentylxanthine, pentoxifylline, and aminophylline. Aminophylline is theophylline compound with 1,2-ethylenediamine (2:1) dihydrate. Generally, the xanthine derivative is added at a concentration in the culture from about 0.0005 to about 25 millimolar, optionally from about 0.001 to about 10 millimolar, from about 0.005 to about 5 millimolar, or from about 0.01 to about 3 millimolar. The optimal concentration of the xanthine derivative will vary depending upon its activity and the cell line, and can be determined by those skilled in the art using the guidance provided herein.

The xanthine derivative can be dissolved in any appropriate solvent. For example, 3-isobutyl-1-methylxanthine (IBX) can be dissolved in water, but must to be heated to almost the boiling point. Alternatively, IBX can be dissolved in the solvents DMSO (dimethylsulfoxide), DMF (dimethylformamide), or DMA (dimethylacetamide). IBX can also be easily dissolved as a 100 millimolar stock solution in 0.5 M NaOH. Dilutions of this stock solution can be added to the induction media as it is being prepared (pre-sterile) and the effects of the NaOH should be inconsequential since base must often be added to raise the pH of the medium to 7.0.

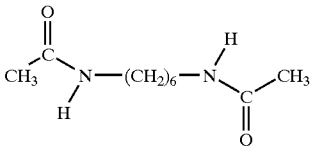
Many of the xanthine derivatives for use in the invention are cAMP phosphodiesterase inhibitors. Thus, in addition to using xanthine derivatives that are cAMP phosphediesterase inhibitors, it is believed that cAMP phosphodiesterase inhibitors that are not xanthine derivatives could also be used to induce polypeptide production in alternative methods of the invention. Examples of such inducers include but are not limited to imidazopyrimidine, pyrazolopyridine, etazolate, pyrazoloquinoline, and triazoloquinazoline (Pflugers Archiv 407: S31, 1986). Other examples cAMP phosphodiesterase inhibitors can be found in U.S. Pat. No. RE37,234, which is incorporated by reference herein.

The hybrid polar compounds, the use of which is encompassed by the invention, can have two polar groups separated by a non-polar carbon chain, such as those described in Richon et al. (1998), Proc. Natl. Acad. Sci. 95: 3003–07, Marks et al. (1994), Proc. Natl. Acad. Sci. 91: 10251–54, U.S. Pat. Nos. 5,055,608 and 6,087,367. The hybrid polar compounds of the invention may have the property of

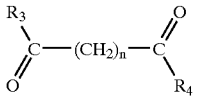
inducing one or more changes characteristic of a terminally differentiated state of the host cells. These compounds include those with the structure:



$R_1$ and $R_2$ can be the same as or different from each other. $R_1$ and $R_2$ can each be a carbonyl group to which a hydrogen atom, a hydroxyl group, a substituted or unsubstituted, branched or unbranched alkyl, alkenyl, cycloalkyl, aryl, alkynl, allenyl, allyl, alkyloxy, aryloxy, arylalkyloxy, which contains 12 or fewer carbon atoms, or pyridine group, may also be attached. The "n" can be an integer from about four to about eight. Specifically, HMBA is included within this class of hybrid polar compounds, and its structure is:



The present invention further encompasses the use of hybrid polar compounds with the following structure:



$R_3$ and $R_4$ can be the same as or different from each other. When $R_3$ and $R_4$ are the same, each is a substituted or unsubstituted arylamino, cycloalkylamino, pyridineamino, piperidino, 9-purine-6-amine, or thiozoleamino group containing 12 or fewer carbon atoms. Where $R_3$ and $R_4$ are different, $R_3$ is equal to $R_5$—N—$R_6$, where $R_5$ and $R_6$ are the same as or different from each other and are a hydrogen atom, a hydroxyl group, a substituted or unsubstituted, branched or unbranched alkyl, alkenyl, cycloalkyl, aryl, alkynl, allenyl, allyl, alkyloxy, aryloxy, arylalkyloxy, or pyridine group, which contains 12 or fewer carbon atoms, or $R_5$ and $R_6$ bond together to form a piperidine group, and $R_4$ is a hydroxylamino, hydroxyl, amino, alkylamino, dialkylamino, or alkyloxy group, which contains 12 or fewer carbon atoms. The "n" is an integer from about four to about eight.

The invention further embraces the use of all compounds disclosed in U.S. Pat. No. 6,087,367, U.S. Pat. No. 5,055,608, Richon et al., supra, and Marks et al., supra. In some of these, the apolar carbon chain may be shorter than 4 carbons and longer than 8 carbons, and it may be interrupted by aromatic groups, apolar groups, and/or polar groups.

If HMBA is used, it can be added at concentrations from about 0.1 millimolar to about 20 millimolar, optionally, between about 0.1 millimolar and about 5 millimolar. Other hybrid polar compounds may be active at lower or higher concentrations. The optimal concentration for a particular hybrid polar compound will vary depending on its activity and the cell line in which it is used and can be determined by one of skill in the art using routine methods and the guidance provided herein. For example, compounds such as suberoylanilide hydroxamic acid or m-carboxycinnamic acid bishydroxamide can be used at concentrations about one thousand fold lower than those required for HMBA, from about 0.01 micromolar to about 10 micromolar. See Richon et al., supra. Concentrations of hybrid polar compounds required to induce cell differentiation as disclosed in Marks et al. (supra) and Richon et al. (supra) can be used as a guide for determining the concentration of a hybrid polar compound required to enhance polypeptide production. Determination of the concentration needed for a specific hybrid polar compound used in a specific cell line can be done using routine methods as described herein and the guidance provided in Richon et al. (supra) and Marks et al. (supra).

The alkanoic acids for use in the invention include the selected acid and/or a corresponding salt. The acids include straight or branched chain, saturated or unsaturated alkanoic acids or salts thereof. An alkanoic acid generally comprises from one to ten carbon atoms. Examples of alkanoic acids contemplated by the invention are pentanoic acid, butyric acid, isobutyric acid, propionic acid, and acetic acid. Concentrations for alkanoic acids encompassed by the invention range from about 0.05 millimolar to about 10 millimolar, optionally from about 0.1 millimolar to about 2 millimolar. Appropriate concentrations of alkanoic acids will vary depending upon their activity and the cell line and can be determined by one of skill in the art using routine methods and the guidance provided herein. An exemplary salt of butyric acid is sodium butyrate. Appropriate salts of the alkanoic acids described above include those comprising sodium, potassium, or ammonium groups, among others.

Particularly preferred polypeptides for expression are polypeptide-based drugs, also known as biologics. Preferably, the polypeptides are secreted as extracellular products. The polypeptide being produced can comprise part or all of a polypeptide that is identical or substantially similar to a naturally-occurring polypeptide, and/or it may, or may not, be a recombinant fusion polypeptide. Optionally, the polypeptide may be a human polypeptide, a fragment thereof, or a substantially similar polypeptide that is at least 15 amino acids in length. It may comprise a non-antibody polypeptide and/or an antibody. It may be produced intracellularly or be secreted into the culture medium from which it can be recovered. It may or may not be a soluble polypeptide.

The polypeptide being produced can comprise part or all of a polypeptide that is identical or substantially similar to a naturally-occurring polypeptide, and/or it may, or may not, be a recombinant fusion polypeptide. It may comprise a non-antibody polypeptide and/or an antibody. It may be produced intracellularly or be secreted into the culture medium from which it can be recovered.

The invention can be used to induce the production of just about any polypeptide, and is particularly advantageous for polypeptides whose expression is under the control of a strong promoter, such as for example, a viral promoter, and/or polypeptides that are encoded on a message that has an adenoviral tripartite leader element. Examples of useful expression vectors that can be used to produce proteins are disclosed in International Application WO 01/27299 and in McMahan et al., (1991), EMBO J. 10: 2821, which describes the pDC409 vector. A protein is generally understood to be a polypeptide of at least about 10 amino acids, optionally about 25, 75, or 100 amino acids.

Generally, the methods of the invention arc useful for inducing the production of recombinant polypeptides. Some polypeptides that can be produced with the methods of the invention include polypeptides comprising amino acid sequences identical to or substantially similar to all or part

of one of the following polypeptides: a flt3 ligand (as described in International Application WO 94/28391, incorporated herein by reference), a CD40 ligand (as described in U.S. Pat. No. 6,087,329 incorporated herein by reference), erythropoeitin, thrombopoeitin, calcitonin, leptin, IL-2, angiopoietin-2 (as described by Maisonpierre et al. (1997), Science 277(5322): 55–60, incorporated herein by reference), Fas ligand, ligand for receptor activator of NF-kappa B (RANKL, as described in International Application WO 01/36637, incorporated herein by reference), tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL, as described in International Application WO 97/01633, incorporated herein by reference), thymic stroma-derived lymphopoietin, granulocyte colony stimulating factor, granulocyte-macrophage colony stimulating factor (GM-CSF, as described in Australian Patent No. 588819, incorporated herein by reference), mast cell growth factor, stem cell growth factor (described in e.g. U.S. Pat. No. 6,204,363, incorporated herein by reference), epidermal growth factor, keratinocyte growth factor, megakaryote growth and development factor, RANTES, growth hormone, insulin, insulinotropin, insulin-like growth factors, parathyroid hormone, interferons including α interferons, γ interferon, and consensus interferons (such as those described in U.S. Pat. Nos. 4,695,623 and 4,897471, both of which are incorporated herein by reference), nerve growth factor, brain-derived neurotrophic factor, synaptotagmin-like proteins (SLP 1–5), neurotrophin-3, glucagon, interleukins 1 through 18, colony stimulating factors, lymphotoxin-β, tumor necrosis factor (TNF), leukemia inhibitory factor, oncostatin-M, and various ligands for cell surface molecules ELK and Hek (such as the ligands for eph-related kinases or LERKS). Descriptions of polypeptides that can be produced according to the inventive methods may be found in, for example, *Human Cytokines: Handbook for Basic and Clinical Research. Vol. II* (Aggarwal and Gutterman, eds. Blackwell Sciences, Cambridge, Mass., 1998); *Growth Factors: A Practical Approach* (McKay and Leigh, eds., Oxford University Press Inc., New York, 1993); and *The Cytokine Handbook* (A. W. Thompson, ed., Academic Press, San Diego, Calif., 1991), all of which are incorporated herein by reference.

Other polypeptides that can be produced using the methods of the invention include polypeptides comprising all or part of the amino acid sequence of a receptor for any of the above-mentioned polypeptides, an antagonist to such a receptor or any of the above-mentioned polypeptides, and/or polypeptides substantially similar to such receptors or antagonists. These receptors and antagonists include: both forms of tumor necrosis factor receptor (TNFR, referred to as p55 and p75, as described in U.S. Pat. No. 5,395,760 and U.S. Pat. No. 5,610,279, both of which are incorporated herein by reference), Interleukin-1 (IL-1) receptors (types I and II; described in EP Patent No. 0 460 846, U.S. Pat. No. 4,968,607, and U.S. Pat. No. 5,767,064, all of which are incorporated herein by reference), IL-1 receptor antagonists (such as those described in U.S. Pat. No. 6,337,072, incorporated herein by reference), IL-1 antagonists or inhibitors (such as those described in U.S. Pat. Nos. 5,981,713, 6,096,728, and 5,075,222, all of which are incorporated herein by reference) IL-2 receptors, IL-4 receptors (as described in EP Patent No. 0 367 566 and U.S. Pat. No. 5,856,296, both of which are incorporated by reference), IL-15 receptors, IL-17 receptors, IL-18 receptors, granulocyte-macrophage colony stimulating factor receptor, granulocyte colony stimulating factor receptor, receptors for oncostatin-M and leukemia inhibitory factor, receptor activator of NF-kappa B (RANK, described in WO 01/36637 and U.S. Pat. No. 6,271,349, both of which are incorporated by reference), osteoprotegerin (described in e.g. U.S. Pat. No. 6,015,938, incorporated by reference), receptors for TRAIL (including TRAIL receptors 1, 2, 3, and 4), and receptors that comprise death domains, such as Fas or Apoptosis-Inducing Receptor (AIR).

Other polypeptides that can be produced using the process of the invention include polypeptides comprising all or part of the amino acid sequences of differentiation antigens (referred to as CD polypeptides) or their ligands or polypeptides substantially similar to either of these. Such antigens are disclosed in *Leukocyte Typing VI* (*Proceedings of the VIth International Workshop and Conference*, Kishimoto, Kikutani et al., eds., Kobe, Japan, 1996, which is incorporated by reference). Similar CD polypeptides are disclosed in subsequent workshops. Examples of such antigens include CD22, CD27, CD30, CD39, CD40, and ligands thereto (CD27 ligand, CD30 ligand, etc.). Several of the CD antigens are members of the TNF receptor family, which also includes 41BB and OX40. The ligands are often members of the TNF family, as are 41BB ligand and OX40 ligand. Accordingly, members of the TNF and TNFR families can also be purified using the present invention.

Enzymatically active polypeptides or their ligands can also be produced according to the methods of the invention. Examples include polypeptides comprising all or part of one of the following polypeptides or their ligands or a polypeptide substantially similar to one of these: metalloproteinase-disintegrin family members, various kinases, glucocerebrosidase, superoxide dismutase, tissue plasminogen activator, Factor VIII, Factor IX, apolipoprotein E, apolipoprotein A-I, globins, an IL-2 antagonist, alpha-1 antitrypsin, TNF-alpha Converting Enzyme, ligands for any of the above-mentioned enzymes, and numerous other enzymes and their ligands.

The methods of the invention can also be used to produce antibodies or portions thereof and chimeric antibodies, i.e. antibodies having human constant antibody immunoglobulin domains coupled to one or more murine variable antibody immunoglobulin domain, fragments thereof, or substantially similar proteins. The method of the invention may also be used to produce conjugates comprising an antibody and a cytotoxic or luminescent substance. Such substances include: maytansine derivatives (such as DM1); enterotoxins (such as a Staphlyococcal enterotoxin); iodine isotopes (such as iodine-125); technium isotopes (such as Tc-99m); cyanine fluorochromes (such as Cy5.5.18); and ribosome-inactivating polypeptides (such as bouganin, gelonin, or saporin-S6). The invention can also be used to produce chimeric proteins selected in vitro to bind to a specific target protein and modify its activity such as those described in International Applications WO 01/83525 and WO 00/24782, both of which are incorporated by reference. Examples of antibodies, in vitro-selected chimeric proteins, or antibody/cytotoxin or antibody/luminophore conjugates that can be produced by the methods of the invention include those that recognize any one or a combination of polypeptides including, but not limited to, the above-mentioned proteins and/or the following antigens: CD2, CD3, CD4, CD8, CD11a, CD14, CD18, CD20, CD22, CD23, CD25, CD33, CD40, CD44, CD52, CD80 (B7.1), CD86 (B7.2), CD147, IL-1α, IL-1β, IL-2, IL-3, IL-7, IL-4, IL-5, IL-8, IL-10, IL-2 receptor, IL-4 receptor, IL-6 receptor, IL-13 receptor, IL-18 receptor subunits, PDGF-β and analogs thereof (such as those described in U.S. Pat. Nos. 5,272,064 and 5,149,792), VEGF, TGF, TGF-β2, TGF-β1, EGF receptor (including

those described in U.S. Pat. No. 6,235,883 B1, incorporated by reference) VEGF receptor, hepatocyte growth factor, osteoprotegerin ligand, interferon gamma, B lymphocyte stimulator (BlyS, also known as BAFF, THANK, TALL-1, and zTNF4; see Do and Chen-Kiang (2002), Cytokine Growth Factor Rev. 13(1): 19–25), C5 complement, IgE, tumor antigen CA125, tumor antigen MUC1, PEM antigen, LCG (which is a gene product that is expressed in association with lung cancer), HER-2, a tumor-associated glycoprotein TAG-72, the SK-1 antigen, tumor-associated epitopes that are present in elevated levels in the sera of patients with colon and/or pancreatic cancer, cancer-associated epitopes or polypeptides expressed on breast, colon, squamous cell, prostate, pancreatic, lung, and/or kidney cancer cells and/or on melanoma, glioma, or neuroblastoma cells, the necrotic core of a tumor, integrin alpha 4 beta 7, the integrin VLA-4, B2 integrins, TRAIL receptors 1, 2, 3, and 4, RANK, RANK ligand, TNF-α, the adhesion molecule VAP-1, epithelial cell adhesion molecule (EpCAM), intercellular adhesion molecule-3 (ICAM-3), leukointegrin adhesin, the platelet glycoprotein gp IIb/IIIa, cardiac myosin heavy chain, parathyroid hormone, rNAPc2 (which is an inhibitor of factor VIIa-tissue factor), MHC I, carcinoembryonic antigen (CEA), alpha-fetoprotein (AFP), tumor necrosis factor (TNF), CTLA-4 (which is a cytotoxic T lymphocyte-associated antigen), Fc-γ-1 receptor, HLA-DR 10 beta, HLA-DR antigen, L-selectin, Respiratory Syncitial Virus, human immunodeficiency virus (HIV), hepatitis B virus (HBV), *Streptococcus mutans*, and *Staphlycoccus aureus*.

The invention may also be used to produce all or part of an anti-idiotypic antibody or a substantially similar polypeptide, including anti-idiotypic antibodies against: an antibody targeted to the tumor antigen gp72; an antibody against the ganglioside GD3; an antibody against the ganglioside GD2; or antibodies substantially similar to these.

The methods of the invention can also be used to produce recombinant fusion polypeptides comprising any of the above-mentioned polypeptides. For example, recombinant fusion polypeptides comprising one of the above-mentioned polypeptides plus a multimerization domain, such as a leucine zipper, a coiled coil, an Fc portion of an antibody, or a substantially similar protein, can be produced using the methods of the invention. See e.g. WO94/10308; Lovejoy et al. (1993), Science 259: 1288–1293; Harbury et al. (1993), Science 262: 1401–05; Harbury et al. (1994), Nature 371:80–83; Håkansson et al. (1999), Structure 7:255–64, all of which are incorporated by reference. Specifically included among such recombinant fusion polypeptides are polypeptides in which a portion of TNFR or RANK is fused to an Fc portion of an antibody (TNFR:Fc or RANK:Fc). TNFR:Fc comprises the Fc portion of an antibody fused to an extracellular domain of TNFR, which includes amino acid sequences substantially similar to amino acids 1–163, 1–185, or 1–235 of FIG. 2A of U.S. Pat. No. 5,395,760, which is incorporated by reference. RANK:Fc is described in International Application WO 01/36637, which is incorporated by reference.

Preferably, the polypeptides are expressed under the control of a heterologous control element such as, for example, a promoter that does not in nature direct the production of that polypeptide. For example, the promoter can be a strong viral promoter (e.g., CMV, SV40) that directs the expression of a mammalian polypeptide. The host cell may or may not normally produce the polypeptide. For example, the host cell can be a CHO cell that has been genetically engineered to produce a human polypeptide, meaning that nucleic acid encoding the human polypeptide has been introduced into the CHO cell. Alternatively, the host cell can be a human cell that has been genetically engineered to produce increased levels of a human polypeptide normally present only at very low levels (e.g., by replacing the endogenous promoter with a strong viral promoter). For the production of recombinant polypeptides, an expression vector encoding the recombinant polypeptide can be transferred, for example by transfection or viral infection, into a substantially homogeneous culture of host cells. The expression vector, which can be constructed using the methods of genetic engineering, can include nucleic acids encoding the polypeptide of interest operably linked to suitable regulatory sequences.

The regulatory sequences are typically derived from mammalian, microbial, viral, and/or insect genes. Examples of regulatory sequences include transcriptional promoters, operators, and enhancers, a ribosomal binding site (see e.g. Kozak (1991), J. Biol. Chem. 266:19867–19870), appropriate sequences to control transcriptional and translational initiation and termination, polyadenylation signals (see e.g. McLauchlan et al. (1988), Nucleic Acids Res. 16:5323–33), and matrix and scaffold attachment sites (see Phi-Van et al. (1988), Mol. Cell. Biol. 10:2302–07; Stief et al. (1989), Nature 341:342–35; Bonifer et al. (1990), EMBO J. 9:2843–38). Nucleotide sequences are operably linked when the regulatory sequence functionally relates to the polypeptide coding sequence. Thus, a promoter nucleotide sequence is operably linked to a polypeptide coding sequence if the promoter nucleotide sequence controls the transcription of the coding sequence. A gene encoding a selectable marker is generally incorporated into the expression vector to facilitate the identification of recombinant cells.

Transcriptional and translational control sequences for mammalian host cell expression vectors can be excised from viral genomes. Commonly used promoter and enhancer sequences are derived from polyoma virus, adenovirus 2, simian virus 40 (SV40), and human cytomegalovirus (CMV). For example, the human CMV promoter/enhancer of immediate early gene 1 may be used. See e.g. Patterson et al. (1994), Applied Microbiol. Biotechnol. 40:691–98. DNA sequences derived from the SV40 viral genome, for example, SV40 origin, early and late promoter, enhancer, splice, and polyadenylation sites can be used to provide other genetic elements for expression of a structural gene sequence in a mammalian host cell. Viral early and late promoters are particularly useful because both are easily obtained from a viral genome as a fragment, which can also contain a viral origin of replication (Fiers et al. (1978), Nature 273:113; Kaufman (1990), Meth. in Enzymol. 185:487–511). Smaller or larger SV40 fragments can also be used, provided the approximately 250 bp sequence extending from the Hind III site toward the Bgl I site located in the SV40 viral origin of replication site is included.

In addition, a sequence encoding an appropriate native or heterologous signal peptide (leader sequence) can be incorporated into the expression vector, to promote extracellular secretion of the recombinant polypeptide. The signal peptide will be cleaved from the recombinant polypeptide upon secretion from the cell. The choice of signal peptide or leader depends on the type of host cells in which the recombinant polypeptide is to be produced. Examples of signal peptides that are functional in mammalian host cells include the signal sequence for interleukin-7 (IL-7) described in U.S. Pat. No. 4,965,195, the signal sequence for interleukin-2 receptor described in Cosman et al. (1984), Nature 312:768; the interleukin-4 receptor signal peptide described in EP Patent No. 367,566; the type I interleukin-1

receptor signal peptide described in U.S. Pat. No. 4,968,607; and the type II interleukin-1 receptor signal peptide described in EP Patent No. 0 460 846.

Established methods for introducing DNA into mammalian cells have been described. Kaufman, R. J., *Large Scale Mammalian Cell Culture*, 1990, pp. 15–69. Additional protocols using commercially available reagents, such as the cationic lipid reagents LIPOFECTAMINE™, LIPOFECTAMINE™-2000, or LIPOFECTAMINE™-PLUS (which can he purchased from Invitrogen), can be used to transfect cells. Feigner et al. (1987)., Proc. Natl. Acad. Sci. USA 84:7413–7417. In addition, electroporation or bombardment with microprojectiles coated with nucleic acids can be used to transfect mammalian cells using procedures, such as those in Sambrook et al., *Molecular Cloning: A Laboratory Manual*, 2nd ed. Vol. 1–3, Cold Spring Harbor Laboratory Press (1989) and Fitzpatrick-McElligott (1992), Biotechnology (NY) 10(9): 1036–40. Selection of stable transformants can be performed using methods known in the art, such as, for example, resistance to cytotoxic drugs. Kaufman et al. ((1990), Meth. in Enzymology 185:487–511), describes several selection schemes, such as dihydrofolate reductase (DHFR) resistance. A suitable host strain for DHFR selection can be CHO strain DX-B11, which is deficient in DHFR. Urlaub and Chasin (1980), Proc. Natl. Acad. Sci. USA 77:4216–4220. A plasmid expressing the DHFR cDNA can be introduced into strain DX-B11, and only cells that contain the plasmid can grow in the appropriate selective media. Other examples of selectable markers that can be incorporated into an expression vector include cDNAs conferring resistance to antibiotics, such as G418 and hygromycin B. Cells harboring the vector can be selected on the basis of resistance to these compounds.

Additional control sequences shown to improve expression of heterologous genes from mammalian expression vectors include such elements as the expression augmenting sequence element (EASE) derived from CHO cells (Morris et al., in *Animal Cell Technology*, pp. 529–534 (1997); U.S. Pat. Nos. 6,312,951 B1, 6,027,915, and 6,309,841 B1) and the tripartite leader (TPL) and VA gene RNAs from Adenovirus 2 (Gingeras et al. (1 982), *J. Biol. Chem.* 257:13475–13491). The internal ribosome entry site (IRES) sequences of viral origin allows dicistronic mRNAs to be translated efficiently (Oh and Sarnow (1993), *Current Opinion in Genetics and Development* 3:295–300; Ramesh et al. (1996), *Nucleic Acids Research* 24:2697–2700). Expression of a heterologous cDNA as part of a dicistronic mRNA followed by the gene for a selectable marker (e.g. DHFR) has been shown to improve transfectability of the host and expression of the heterologous cDNA (Kaufman et al. (1990), Methods in Enzymol. 185:487–511). Exemplary expression vectors that employ dicistronic mRNAs are pTR-DC/GFP described by Mosser et al., *Biotechniques* 22:150–161 (1997), and p2A5I described by Morris et al., in *Animal Cell Technology*, pp. 529–534 (1997).

A useful high expression vector, pCAVNOT, has been described by Mosley et al. ((1989), *Cell* 59:335–348). Other expression vectors for use in mammalian host cells can be constructed as disclosed by Okayama and Berg ((1983), Mol. Cell. Biol. 3:280). A useful system for stable high level expression of mammalian cDNAs in C127 murine mammary epithelial cells can be constructed substantially as described by Cosman et al. ((1986), Mol. Immunol. 23:935). A useful high expression vector, PMLSV N1/N4, described by Cosman et al. ((1984), Nature 312:768), has been deposited as ATCC 39890. Additional useful mammalian expression vectors are described in EP Patent No.-A-0 367 566 and WO 01/27299 A1. The vectors can be derived from retroviruses. In place of the native signal sequence, a heterologous signal sequence can be added, such as one of the following sequences: the signal sequence for IL-7 described in U.S. Pat. No. 4,965,195; the signal sequence for IL-2 receptor described in Cosman et al. (Nature 312:768 (1984)); the IL-4 signal peptide described in EP Patent No. 0 367 566; the typed IL-1 receptor signal peptide described in U.S. Pat. No. 4,968,607; and the type II IL-1 receptor signal peptide described in EP Patent No. 0 460 846.

The polypeptides can be produced recombinantly in eukaryotic cells and are preferably secreted by host cells adapted to grow in cell culture. Optionally, host cells for use in the invention are preferably mammalian cells. The cells can be also genetically engineered to express a gene of interest, can be mammalian production cells adapted to grow in cell culture, and/or can be homogenous cell lines. Examples of such cells commonly used in the industry are VERO, BHK, HeLa, CV1 (including Cos), MDCK, 293, 3T3, myeloma cell lines (e.g., NSO, NS1), PC12, WI38 cells, and Chinese hamster ovary (CHO) cells, which are widely used for the production of several complex recombinant polypeptides, e.g. cytokines, clotting factors, and antibodies (Brasel et al. (1996), Blood 88:2004–2012; Kaufman et al. (1988), J.Biol Chem 263:6352–6362; McKinnon et al. (1991), J Mol Endocrinol 6:231–239; Wood et al. (1990), J. Immunol. 145:3011–3016). The dihydrofolate reductase (DHFR)-deficient mutant cell lines (Urlaub et al. (1980), Proc Natl Acad Sci USA 77: 4216–4220, which is incorporated by reference), DXB11 and DG-44, are desirable CHO host cell lines because the efficient DHFR selectable and amplifiable gene expression system allows high level recombinant polypeptide expression in these cells (Kaufman R. J. (1990), Meth Enzymol 185:537–566, which is incorporated by reference). In addition, these cells are easy to manipulate as adherent or suspension cultures and exhibit relatively good genetic stability. CHO cells and recombinant polypeptides expressed in them have been extensively characterized and have been approved for use in clinical commercial manufacturing by regulatory agencies. The methods of the invention can also be practiced using hybridoma cell lines that produce an antibody. Methods for making hybridoma lines are well known in the art. See e.g. Berzofsky et al. in Paul, ed., *Fundamental Immunology, Second Edition*, pp.315–356, at 347–350, Raven Press Ltd., New York (1989). Cell lines derived from the above-mentioned lines are also suitable for practicing the invention.

According to the present invention, a mammalian host cell is cultured under conditions that promote the production of the polypeptide of interest, which can be an antibody or a recombinant polypeptide. Basal cell culture medium formulations are well known in the art. To these basal culture medium formulations the skilled artisan will add components such as amino acids, salts, sugars, vitamins, hormones, growth factors, buffers, antibiotics, lipids, trace elements and the like, depending on the requirements of the host cells to be cultured. The culture medium may or may not contain serum and/or protein. Various tissue culture media, including serum-free and/or defined culture media, are commercially available for cell culture. Tissue culture media is defined, for purposes of the invention, as a media suitable for growth of animal cells, and preferably mammalian cells, in in vitro cell culture. Typically, tissue culture media contains a buffer, salts, energy source, amino acids, vitamins and trace essential elements. Any media capable of supporting

growth of the appropriate eukaryotic cell in culture can be used; the invention is broadly applicable to eukaryotic cells in culture, particularly mammalian cells, and the choice of media is not crucial to the invention. Tissue culture media suitable for use in the invention are commercially available from, e.g., ATCC (Manassas, Va.). For example, any one or combination of the following media can be used: RPMI-1640 Medium, RPMI-1641 Medium, Dulbecco's Modified Eagle's Medium (DMEM), Minimum Essential Medium Eagle, F-12K Medium, Ham's F12 Medium, Iscove's Modified Dulbecco's Medium, McCoy's 5A Medium, Leibovitz's L-15 Medium, and serum-free media such as EX-CELL™ 300 Series (available from JRH Biosciences, Lenexa, Kans., USA), among others, which can be obtained from the American Type Culture Collection or JRH Biosciences, as well as other vendors. When defined medium that is serum-free and/or peptone-free is used, the medium is usually highly enriched for amino acids and trace elements. See, for example, U.S. Pat. Nos. 5,122,469 to Mather et al. and 5,633,162 to Keen et al.

In the methods and compositions of the invention, cells can be grown in serum-free, protein-free, growth factor-free, and/or peptone-free media. The term "serum-free" as applied to media includes any mammalian cell culture medium that does not contain serum, such as fetal bovine serum. The term "insulin-free" as applied to media includes any medium to which no exogenous insulin has been added. By exogenous is meant, in this context, other than that produced by the culturing of the cells themselves. The term "IGF-1-free" as applied to media includes any medium to which no exogenous Insulin-like growth factor-1 (IGF-1) or analog (such as, for example, LongR3, [Ala31], or [Leu24] [Ala31] IGF-1 analogs available from GroPep Ltd. of Thebarton, South Australia) has been added. The term "growth-factor free" as applied to media includes any medium to which no exogenous growth factor (e.g., insulin, IGF-1) has been added. The term "protein-free" as applied to media includes medium free from exogenously added protein, such as, for example, transferrin and the protein growth factors IGF-1 and insulin. Protein-free media may or may not have peptones. The term "peptone-free" as applied to media includes any medium to which no exogenous protein hydrolysates have been added such as, for example, animal and/or plant protein hydrolysates. Eliminating peptone from media has the advantages of reducing lot to lot variability and enhancing processing such as filtration. Chemically defined media are media in which every component is defined and obtained from a pure source, preferably a non-animal source.

The skilled artisan may also choose to use one of the many individualized media formulations that have been developed to maximize cell growth, cell viability, and/or recombinant polypeptide production in a particular cultured host cell. The methods according to the current invention may be used in combination with commercially available cell culture media or with a cell culture medium that has been individually formulated for use with a particular cell line. For example, an enriched medium that could support increased polypeptide production may comprise a mixture of two or more commercial media, such as, for instance, DMEM and Ham's F12 media combined in ratios such as, for example, 1:1, 1:2, 1:3, 1:4, 1:5, 1:6, 1:7, 1:8, or even up to 1:15 or higher. Alternatively or in addition, a medium can be enriched by the addition of nutrients, such as amino acids or peptone, and/or a medium (or most of its components with the exceptions noted below) can be used at greater than its usual, recommended concentration, for example at 2×, 3×, 4×, 5×, 6×, 7×, 8×, or even higher concentrations. As used herein, "1×" means the standard concentration, "2×" means twice the standard concentration, etc. In any of these embodiments, medium components that can substantially affect osmolarity, such as salts, cannot be increased in concentration so that the osmolarity of the medium falls outside of an acceptable range. Thus, a medium may, for example, be 8× with respect to all components except salts, which can be present at only 1×. An enriched medium may be serum free and/or protein free. Further, a medium may be supplemented periodically during the time a culture is maintained to replenish medium components that can become depleted such as, for example, vitamins, amino acids, and metabolic precursors. As is known in the art, different media and temperatures may have somewhat different effects on different cell lines, and the same medium and temperature may not be suitable for all cell lines.

Suitable culture conditions for mammalian cells are known in the art. See e.g. Animal cell culture: A Practical Approach, D. Rickwood, ed., Oxford university press, New York (1992). Mammalian cells may be cultured in suspension or while attached to a solid substrate. Furthermore, mammalian cells may be cultured, for example, in fluidized bed bioreactors, hollow fiber bioreactors, roller bottles, shake flasks, or stirred tank bioreactors, with or without microcarriers, and operated in a batch, fed batch, continuous, semi-continuous, or perfusion mode.

The methods according to the present invention may be used to improve the production of recombinant polypeptides in both single phase and multiple phase culture processes. In a single phase process, cells are inoculated into a culture environment and the disclosed methods are employed during the single production phase. In a multiple stage process, cells arc cultured in two or more distinct phases. For example cells may be cultured first in a growth phase, under environmental conditions that maximize cell proliferation and viability, then transferred to a production phase, under conditions that maximize polypeptide production. The growth and production phases may be preceded by, or separated by, one or more transition phases. In multiple phase processes the methods according to the present invention are employed at least during the production phase. A growth phase may occur at a higher temperature than a production phase. For example, a growth phase may occur at a first temperature from about 35° C. to about 38° C., and a production phase may occur at a second temperature from about 29° C. to about 36° C., optionally from about 30° C. to about 33° C. Chemical inducers of polypeptide production, such as, for example, caffeine, butyrate, and HMBA, may be added at the same time as, before, and/or after a temperature shift. If inducers are added after a temperature shift, they can be added from one hour to five days after the temperature shift, optionally from one to two days after the temperature shift.

After induction using the methods of the invention, the resulting expressed polypeptide can then be collected. In addition, the polypeptide can purified, or partially purified, from such culture or component (e.g., from culture medium or cell extracts or bodily fluid) using known processes. By "partially purified" means that some fractionation procedure, or procedures, have been carried out, but that more polypeptide species (at least 10%) than the desired polypeptide is present. By "purified" is meant that the polypeptide is essentially homogeneous, i.e., less than 1% contaminating polypeptides are present. Fractionation procedures can include but are not limited to one or more steps of filtration, centrifugation, precipitation, phase separation,

affinity purification, gel filtration, ion exchange chromatography, hydrophobic interaction chromatography (HIC; using such resins, as phenyl ether, butyl ether, or propyl ether), HPLC, or some combination of above.

For example, the purification of the polypeptide can include an affinity column containing agents which will bind to the polypeptide; one or more column steps over such affinity resins as concanavalin A-agarose, heparin-TOYOPEARL® (Toyo Soda Manufacturing Co. Ltd., Japan) or Cibacrom blue 3GA SEPHAROSE® (Pharmacia Fine Chemicals, Inc., New York); one or more steps involving elution; and/or immunoaffinity chromatography. The polypeptide can be expressed in a form that facilitates purification. For example, it may be expressed as a fusion polypeptide, such as those of mialtose binding polypeptide (MBP), glutathione-S-transferase (GST), or thioredoxin (TRX). Kits for expression and purification of such fusion polypeptides are commercially available from New England BioLab (Beverly, Mass.), Pharmacia (Piscataway, N.J.) and InVitrogen, respectively. The polypeptide can be tagged with an epitope and subsequently purified by using a specific antibody directed to such epitope. One such epitope (FLAGS is commercially available from Kodak (New Haven, Conn.). It is also possible to utilize an affinity column comprising a polypeptide-binding protein, such as a monoclonal antibody to the recombinant polypeptide, to affinity-purify expressed polypeptides. Other types of affinity purification steps can be a Protein A or a Protein G column, which affinity agents bind to proteins that contain Fc domains. Polypeptides can be removed from an affinity column using conventional techniques, e.g., in a high salt elution buffer and then dialyzed into a lower salt buffer for use or by changing pH or other components depending on the affinity matrix utilized, or can be competitively removed using the naturally occurring substrate of the affinity moiety.

The desired degree of final purity depends on the intended use of the polypeptide. A relatively high degree of purity is desired when the polypeptide is to be administered in vivo, for example. In such a case, the polypeptides are purified such that no polypeptide bands corresponding to other polypeptides are detectable upon analysis by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). It will be recognized by one skilled in the pertinent field that multiple bands corresponding to the polypeptide can be visualized by SDS-PAGE, due to differential glycosylation, differential post-translational processing, and the like. Optionally, the polypeptide of the invention is purified to substantial homogeneity, as indicated by a single polypeptide band upon analysis by SDS-PAGE. The polypeptide band can be visualized by silver staining, Coomassie blue staining, or (if the polypeptide is radiolabeled) by autoradiography.

The invention also optionally encompasses further formulating the polypeptides. By the term "formulating" is meant that the polypeptides can be buffer exchanged, sterilized, bulk-packaged, and/or packaged for a final user. For purposes of the invention, the term "sterile bulk form" means that a formulation is free, or essentially free, of microbial contamination (to such an extent as is acceptable for food and/or drug purposes), and is of defined composition and concentration. The term "sterile unit dose form" means a form that is appropriate for the customer and/or patient administration or consumption. Such compositions can comprise an effective amount of the polypeptide, in combination with other components such as a physiologically acceptable diluent, carrier, or excipient. The term "physiologically acceptable" means a non-toxic material that does not interfere with the effectiveness of the biological activity of the active ingredient(s).

Formulations suitable for administration include aqueous and non-aqueous sterile injection solutions which may contain anti-oxidants, buffers, bacteriostats, and solutes which render the formulation isotonic with the blood of the recipient; and aqueous and non-aqueous sterile suspensions which may include suspending agents or thickening agents. The polypeptides can be formulated according to known methods used to prepare pharmaceutically useful compositions. They can be combined in admixture, either as the sole active material or with other known active materials suitable for a given indication, with pharmaceutically acceptable diluents (e.g., saline, Tris-HCl, acetate, and phosphate buffered solutions), preservatives (e.g., thimerosal, benzyl alcohol, parabens), emulsifiers, solubilizers, adjuvants, and/or carriers. Suitable formulations for pharmaceutical compositions include those described in *Remington's Pharmaceutical Sciences*, 16th ed. 1980, Mack Publishing Company, Easton, Pa. In addition, such compositions can be complexed with polyethylene glycol (PEG), metal ions, or incorporated into polymeric compounds such as polyacetic acid, polyglycolic acid, hydrogels, dextran, etc., or incorporated into liposomes, microemulsions, micelles, unilamellar or multilamellar vesicles, erythrocyte ghosts or spheroblasts. Suitable lipids for liposomal formulation include, without limitation, monoglycerides, diglycerides, sulfatides, lysolecithin, phospholipids, saponin, bile acids, and the like. Preparation of such liposomal formulations is within the level of skill in the art, as disclosed, for example, in U.S. Pat. Nos. 4,235,871, 4,501,728, 4,837,028, and 4,737,323. Such compositions will influence the physical state, solubility, stability, rate of in vivo release, and rate of in vivo clearance, and are thus chosen according to the intended application, so that the characteristics of the carrier will depend on the selected route of administration. Sustained-release forms suitable for use include, but are not limited to, polypeptides that are encapsulated in a slowly-dissolving biocompatible polymer (such as the alginate microparticles described in U.S. Pat. No. 6,036,978), admixed with such a polymer (including topically applied hydrogels), and or encased in a biocompatible semi-permeable implant.

The invention having been described, the following examples are offered by way of illustration, and not limitation.

## EXAMPLE 1

### Comparison of the Inducing Activity of Caffeine and Butyrate at 31° C.

In this experiment, caffeine (at concentrations from 0.5 to 2.0 mM) was compared to sodium butyrate for its ability to induce expression of a recombinant polypeptide. A CHO cell production line genetically engineered to express TNFR:Fc (cell line #5) was used to test the effectiveness of caffeine as an inducing agent. CHO cells were grown in spinner flasks at 37° C. using serum-free growth medium containing methotrexate. When the appropriate cell mass was obtained, the cells were placed into induction conditions by a five minute centrifugation at 1000×g, followed by replacement of the growth medium with serum-free medium without methotrexate. The cells, at initial cell densities of $2\times10^6$ cells/ml in 20 ml, were placed in 125 ml plastic Erlenmeyer flasks with plug seal caps and placed on shaker platforms in incubators set to the appropriate temperatures. Cell viability and number were monitored by haemocytometer counting using trypan blue dye. Recombinant polypeptide titers were assessed by ELISA-based assays.

For this cell line, 0.2 mM was known to be the optimal concentration of sodium butyrate for induction.

Accordingly, 0.2 mM sodium butyrate was compared against the inducing effects of 0.5, 1.0, and 2.0 mM caffeine. A flask containing no inducing compound was also included. The shaker flasks were incubated in this induction phase for 5 days at 31° C. in incubators without carbon dioxide control.

After 5 days in culture, cell viabilities for all of the tested conditions were very similar and ranged between 75 and 85%. The highest relative protein titer (in μg/ml), which was about 1.15 times the titer of the control culture without inducers, and relative productivity (in μg protein/$10^6$ cells/day), which was about 1.3 times the productivity of the control culture, was exhibited by the cells that were induced with 1 mM caffeine. Cells induced with 0.2 mM butyrate produced about 1.11 times the total protein (in μg/ml) produced by the control culture at a rate (in μg protein/$10^6$ cells/day) that was about 1.07 times the rate of control cultures. Similar protein titers were observed in the cells induced with 0.5 mM caffeine, although these cultures had slightly higher rates of production. At caffeine concentrations of 2 mM, protein titer was similar to that observed with no inducing agent, although the rate of productivity per cell was higher.

These results indicate that caffeine can be used as an inducing agent and can induce product titers equal or exceeding those observed using sodium butyrate as an inducing agent. In addition, further experimental data was obtained which indicated that recombinant polypeptide produced using caffeine was equal in product quality (e.g., glycosylation, folding, and amino acid composition) to that produced using sodium butyrate.

## EXAMPLE 2

### Induction of Recombinant Polypeptide Expression in Cell Line #9

In this experiment, the effect of caffeine (at concentrations from 0 to 1.4 mM) on the induction of expression of a different recombinant polypeptide, a soluble form of the IL-1 receptor type II, in a second CHO cell line (cell line #9) was examined.

CHO cells were grown in spinner flasks at 37° C. using serum-free growth medium containing methotrexate. When the appropriate cell mass was obtained, spent medium was removed by a five minute centrifugation at 1000×g and replaced with production medium without methotrexate. The cells, with initial cell densities of $2\times10^6$ cells/ml in 20 ml, were placed in 125 ml plastic Erlenmeyer flasks with plug seal caps. The following caffeine concentrations were tested: 0, 0.6, 0.8, 1.0, 1.2, and 1.4 mM caffeine. The flasks were then incubated in this induction phase for 5 days at 31° C. in incubators without carbon dioxide control. Cell viability and number were monitored by haemocytometer counting using trypan blue dye. Recombinant polypeptide titers were assessed by ELISA-based assays. Each induction assessment experiment was carried out for 5 days.

After 5 days in culture, cell viability for most of the tested conditions was similar and averaged around 85%. FIG. **1**. For the flask induced with 1.4 mM caffeine, 67% cell viability was observed after 5 days. Similar protein titers were observed using 0.6 mM, 0.8 mM, and 1.0 mM caffeine, that is, about 350 μg/mL, which is equal to the titer observed for 0.5 mM butyrate. FIG. **2**. Since 0.6 mM is the lowest caffeine concentration tested, these data do not exclude the possibility that even lower concentrations of caffeine might give equal or better results. The highest productivity (in μg protein/$10^6$ cells/day) observed for a caffeine-induced culture was in the 0.8 mM caffeine culture. FIG. **3**. At higher levels of caffeine, i.e., 1.2 and 1.4 mM, protein titers were comparable to the negative control (no inducing agent), although productivity on a per cell basis was somewhat higher. FIGS. **2** and **3**.

## EXAMPLE 3

### Induction Of Recombinant Polypeptide Expression In Cell Line #60

In this experiment, the use of caffeine to induce recombinant production from a third CHO cell line (cell line #60) expressing a third recombinant product, a human antibody that recognizes epidermal growth factor receptor, was analyzed. For this cell line, the inducing effects of 0, 0.5, 1.0, 1.5, and 2.0 mM caffeine were tested, and the experiment was conducted as in the previous experiment except that the induction phase was performed at 36° C.

At day 5, the flask of cells with no inducer and the flask of cells induced with 0.5 mM caffeine exhibited the highest cell viabilities (about 76%) of all the conditions. Viabilities of cultures containing 1.0 mM and 1.5 mM caffeine were about 68% and 60%, respectively. Cultures containing 0.75 mM butyrate or 2.0 mM caffeine were about 51% viable. Thus viability, overall, was lower than that seen in cell line #9 at 5 days, an effect that might be attributed to a variety of factors including the difference in temperature and/or cell line differences. A clear dose-response was observed with higher caffeine concentrations leading to lower cell viabilities.

The highest day 5 protein titer was observed in cells induced by 0.5 mM caffeine (305 μg/ml), which was about 111% of the titer of the control culture with no inducer. Generally, the titer of recombinant polypeptide was less as caffeine concentrations increased above 0.5 mM. Productivity (in μg protein/$10^6$ cells/day) appeared to be linked to caffeine concentration, with the highest productivity obtained from cells induced with 2.0 mM caffeine and a lower level of productivity obtained from the cells induced with lower caffeine concentrations. Since a 0.5 mM was the lowest caffeine concentration tested as well as the most effective concentration tested for the induction of protein production, these data do not exclude the possibility that a lower concentration of caffeine might be equally or more effective as an inducer of cell line #60 incubated at 36° C.

This experiment, along with those described in Examples 1 and 2, demonstrates that the ability of caffeine to induce recombinant polypeptide expression is not cell line-specific and that favorable cell viability is maintained in caffeine's presence. In addition, caffeine can be used in an induction or production phase implemented at temperatures from 31° C. to 36° C. However, these data also indicate differences between cell lines in how effectively caffeine induces the synthesis of a recombinant protein. For example, induction of cell line #9 with caffeine is more effective than induction of cell line #60. Compare Example 2 and FIG. **2** to Example 3.

## EXAMPLE 4

### Optimization of Induction for Cell Line #60

The purpose of this experiment was to test ranges of temperature and caffeine concentrations in shake flasks in order to optimize the induction conditions for the cell line #60.

Materials and Methods. Twelve shaker flasks were set up under the conditions described in Table 1.

TABLE 1

Caffeine Concentrations and Temperatures of Samples

| Flask Number | Temperature (° C.) | Caffeine (mM) |
|---|---|---|
| 1 | 36 | 0 |
| 2 | 36 | 0.5 |
| 3 | 36 | 1.0 |
| 4 | 36 | 1.5 |
| 5 | 36 | 2.0 |
| 6 | 36 | 2.5 |
| 7 | 37 | 0 |
| 8 | 37 | 0.5 |
| 9 | 37 | 1.0 |
| 10 | 37 | 1.5 |
| 11 | 37 | 2.0 |
| 12 | 37 | 2.5 |

Cells were collected via centrifugation from a spinner culture of cell line #60 ($26.85\times10^5$ cells/ml, 95.2% viable) and inoculated into a 575 ml spinner flask at $2\times10^6$ cells/ml in serum-free production medium. The culture was then aliquoted into twelve shake flasks. Caffeine was added according to the experimental plan described in Table 1. The shake flasks were incubated at the designated temperatures for 7 days. Samples were taken on days 3, 5 and 7. Cell density and viability were measured using an automated system of cell counting that employs trypan blue staining to determine viability (the Cell Density Examination System or Cedex, developed by innovatis GmbH, Bielefeld, Germany). Glucose and lactate measurements were taken with the Yellow Springs Instruments 2700 Select (available from Yellow Springs Instruments, Yellow Springs, Ohio, USA). Glucose was added on demand to maintain a concentration of >2 g/l. $CO_2$ and external pH were measured using the Ciba-Corning 248 blood gas analyzer (available from Bayer Diagnostics, Tarryton, N.Y., USA). Protein titers were determined via a pre-purification of the antibody on a Protein A column followed by a measurement of the absorbance of the protein bound and eluted from the column at 280 nanometers. Cumulative viable cell densities (CVCDs) were calculated as follows: the CVCD for day 1 is the number of viable cells per milliliter of culture as measured on day 1; the CVCD for day 2 is the number of viable cells per milliliter of culture as measured on day 2 plus the number of viable cells per milliliter of culture as measured on day 1; the CVCD for day 3 is the number of viable cells per milliliter of culture as measured on day 3 plus the numbers of viable cells per milliliter of culture measured on days 1 and 2; and CVCDs for subsequent days are calculated in a similar manner.

Results. Higher CVCDs were achieved in the presence of little or no caffeine. Lower temperature, i.e., 36° C. rather than 37° C. and lower levels of caffeine resulted in higher final viability. Caffeine at 2.5 mM resulted in cell death and termination of the cultures. Over the rest of the concentration range tested, increased levels of caffeine resulted in increased cumulative specific productivity (Cum Qp), with the highest level being almost 30 $\mu g/10^6$ cells/day. Cultures containing the highest levels of caffeine resulting in viable cultures (2 mM), while having a high Cum Qp, had a low CVCD, indicating that 2 mM caffeine decreased cell viability but increased the productivity of remaining viable cells. However, protein titers of cultures induced with 2 mM caffeine were lower than for uninduced cultures at 7 days at both temperatures.

The highest protein titers resulted at the low to intermediate levels of caffeine for both temperatures. The highest day 7 titer was observed in the culture grown at 36° C. in the presence of 0.5 mM caffeine, and its titer was about 124% of the titer seen in a control culture grown at 36° C. for 7 days without inducers. Day 7 titers of 36° C. cultures grown in the presence of 1.0 mM and 1.5 mM caffeine were about 116% and 111% of control levels, respectively. The day 7 titers of cultures grown at 37° C. in the presence of 0.5 mM, 1.0 mM, and 1.5 mM caffeine were about 110%, 112%, and 109%, respectively, of the 37° C. no inducer control culture. Together, these data indicate that induction of cell line #60 was more effective at 36° C. than it was at 37° C. Day 7 titers of control cultures without inducers grown at 36° C. and 37° C. were comparable. Thus, as in Example 3, the lowest concentration of caffeine tested led to the highest protein titers at 36° C., suggesting the possibility that even lower concentrations might produce equal or higher titers.

In summary, induction with caffeine increased specific productivity and titer at both 36° C. and 37° C. Titers were modestly higher at 36° C. than at 37° C. despite lower Cum Qp values because of higher CVCDs and viability at the lower temperature. Based on cell performance and productivity, caffeine can be used to induce production from this cell line.

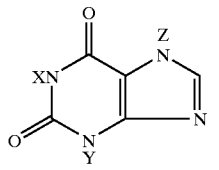
## EXAMPLE 5

### Induction Effects for Compounds Related to Caffeine in Cell Line #9

Since the above experiments showed that caffeine as an inducing agent increased titers of recombinant polypeptide between about 9% and about 67%, additional experiments were performed with other xanthine derivatives to test their inducing ability. Based upon the structure of xanthine, a variety of compounds were modeled and chosen for testing. These include 3-isobutyl-1-methylxanthine, theophylline, theobromine, pentoxifylline, and aminophylline, the structures of which are illustrated below.

TABLE 2

Xanthine Derivatives




| Compound | X | Y | Z |
|---|---|---|---|
| caffeine | methyl | methyl | methyl |
| 3-isobutyl-1-methylxanthine (IBX) | methyl | isobutyl | hydrogen |
| theophylline | methyl | methyl | hydrogen |
| theobromine | hydrogen | methyl | methyl |
| pentoxifylline | 5-oxyhexyl | methyl | methyl |

Aminophylline is theophylline compound with 1,2-ethylenediamine (2:1) dihydrate.

These xanthine derivatives, including some combinations, were tested on cell line #9 in a shake-flask format (20 ml in 125 ml shake flasks) as described above for Examples 1 and 2. To dissolve 3-isobutyl-1-methylxanthine (IBX), it was solubilized in water heated to almost the boiling point, and quickly added to the flasks before it precipitated. Alternatively, IBX was dissolved in DMF. The induction phase of the cell culture was carried out for 6 days at 31° C.,

and samples were removed for analysis at 3 day and 6 day timepoints. The protein titers from each shake flask are shown in Table 3.

TABLE 3

Recombinant Polypeptide Titer Under Various Inducing Conditions

| Condition | Titer (μg/ml) Day 3 | Titer (μg/ml) Day 6 |
|---|---|---|
| 0.6 mM caffeine + 0.5 mM butyrate | 120 | 280 |
| 0.1 mM theobromine | 90 | 270 |
| 0.5 mM theobromine | 100 | 260 |
| 1 mM theobromine | 100 | 260 |
| 0.1 mM aminophylline | 110 | 260 |
| 0.5 mM aminophylline | 120 | 250 |
| 1 mM aminophylline | 100 | 200 |
| 0.1 mM pentoxyphylline | 110 | 320 |
| 0.5 mM pentoxyphylline | 130 | 380 |
| 1 mM pentoxyphylline | 130 | 400 |
| 0.3% DMF + 0.5 mM IBX | not determined | 340 |
| 0.3% DMF | 120 | 330 |
| 0.5 mM IBX | 150 | 420 |
| 0.5 mM IBX + 0.6 mM caffeine | 130 | 370 |
| 0.1 mM IBX + 0.6 mM caffeine | 140 | 390 |
| 0.1 mM IBX + 0.6 mM caffeine + 0.5 mM butyrate | 130 | 280 |
| 0.1 mM IBX + 0.6 mM caffeine + 0.3% DMF | 150 | 390 |
| 0.2 mM caffeine | 150 | 400 |
| 0.6 mM caffeine | 120 | 320 |
| 0.5 mM butyrate | 100 | 220 |
| NO INDUCER | 100 | 290 |

Several conclusions can be made from this data. Production from the flask induced with 0.5 mM IBX was even better than caffeine, and the 3 flasks containing pentoxyphylline also gave promising results. Titers of pentoxyphylline-induced cultures increased with increasing dose and were higher than the tier of the no inducer control culture. Additionally, some combinations of different xanthine derivatives, as well as different xanthine derivatives with other inducing agents (e.g., butyrate and/or DMF) yielded protein titers above control levels. Theobromine and aminophylline did not induce protein titers above that seen in the no inducer control culture. The highest protein liters obtained when caffeine was used as an inducer were obtained at the lowest concentration tested, that is, 0.2 mM caffeine. As explained above, such a result leaves open the possibility that even lower concentrations of caffeine may be effective. Finally, unlike in Example 2 (FIG. **2**), butyrate does not induce increased protein titer over that seen in a culture with no inducer.

## EXAMPLE 6

### Induction of Cell Line #60 by Various Inducing Agents at 37° C.

This experiment was done in shaken Erlenmeyer flasks as described above in Examples 1 and 2, except that the flasks were incubated for 5 days at 37° C., rather than at 31° C., and cell line #60 was used. As a control, one flask without inducers was grown at 31° C. The titer of recombinant polypeptide in the medium was assayed after 5 days. Xanthine derivatives tested included caffeine (at 0.5 mM), theobromine (at 0.1 mM, 0.5 mM, and 1.0 mM), 3-isobutyl-1-methylxanthine (IBX, at 0.05 mM, 0.1 mM, and 0.15 mM), and pentoxyphylline (at 0.1 mM, 0.5 mM, and 1.0 mM). In addition, butyrate, some combinations of inducers, and the non-xanthine compound papaverine were tested.

The 31° C. control culture yielded low protein titers compared to the 37° C. control culture, probably due to the preference of cell line #60 for higher temperatures. Theobromine at a concentration of 0.1 mM increased protein titer over that seen in the 37° C. control culture, but was counterproductive at higher concentrations (0.5 mM and 1.0 mM). Neither caffeine, IBX, or pentoxyphylline increased protein titers above that seen in a control culture with no inducers. Protein titer was inversely proportional to theobromine, IBX, and pentoxyphylline concentrations in the ranges tested. Interestingly, cell line #9 (Table 3, Example 5) showed increased protein titers with increasing pentoxyphylline concentrations within this same range, highlighting the variability in the responses of different cell lines incubated at different temperatures to inducing agents. As in other experiments (see Example 3), 0.5 mM caffeine appears to be a better inducer than 0.5 mM butyrate for cell line #60, although both failed to increase protein titer over that seen in the control culture with no inducing agent in this experiment. In a previous experiment, caffeine had induced slightly higher protein production than that seen in a control culture at 37° C. at day 7 (about 110% of the titer seen in the control culture), although a greater induction was observed at 36° C. Example 4. The failure of caffeine to induce increased protein production in this experiment may be explained by a variety of factors such as experimental variability, the small size of the positive effect at 37° C. in cell line #60, and/or the possibility that 0.5 mM may not be an optimum caffeine concentration for induction of cell line #60 at 37° C.

## EXAMPLE 7

### Production of RANK:Fc in the Presence of Varying Amounts of HMBA

Nucleic acids encoding RANK:Fc inserted into a suitable vector (as described in International Application WO 01/36637) were introduced into CHO cells. About 2 million cells from a stably transformed line propagated at 37° C. were inoculated into 20 milliliters of medium at 31° C., either without HMBA or in the presence of varying concentrations of HMBA, as indicated in Table 4. Cells were grown for a total of 5 days in shaker flasks. Thereafter, all medium was harvested. The number of cells present in the culture was determined by staining with trypan blue and counting the cells in a hemocytometer. The titer of RANK:Fc per milliliter of harvested medium was determined by purifying RANK:Fc by Protein A high performance liquid chromatography (HPLC) and subsequently measuring absorbance at 280 nanometers. An average number of cells in the culture was calculated by averaging the starting and ending cell numbers. Specific productivity was determined from the total number of micrograms of RANK:Fc produced, an average cell number (calculated as described above), and the number of days of growth. Data from this experiment are shown in Table 4.

TABLE 4

Effects of Varying Concentrations of HMBA on Protein Titer and Specific Productivity

| HMBA concentration (mM) | Specific productivity ($\mu g/10^6$ cells/day) | Titer of RANK:Fc (μg/ml) |
|---|---|---|
| 0 | 17.1 | 272 |
| 0.1 | 19.1 | 243 |
| 0.5 | 19.5 | 347 |
| 2.0 | 23.9 | 444 |

These data indicate that the addition of HMBA at concentrations of 0.5 or 2.0 mM had positive effects on polypeptide production and specific productivity.

EXAMPLE 8

Production of RANK:Fc in the Presence of HMBA, Caffeine, and/or Butyric Acid

Nucleic acids encoding RANK:Fc inserted into a suitable vector (as described in International Application WO 01/36637) were introduced into CHO cells. About 2 million cells from a stably transformed line propagated at 37° C. were inoculated into 20 milliliters of serum-free medium at 31° C. without inducers or in the presence of of HMBA and/or caffeine and/or butyric acid, as indicated in Table 5. Cells were grown for a total of 5 days in a shaker flask. Thereafter, all medium was harvested. The number of cells present in the culture, the titer of RANK:Fc per milliliter of harvested medium, and specific productivity were determined as described above in Example 7. Data from this experiment are shown in Table 5.

TABLE 5

Effects of Caffeine, HMBA, and Butyric Acid Singly and In Combination on Protein Titer and Specific Productivity

| Inducer | Specific productivity ($\mu g/10^6$ cells/day) | Titer of RANK:Fc ($\mu g$/ml) |
|---|---|---|
| None | 14.8 | 261 |
| HMBA (2 mM) | 22.8 | 410 |
| Caffeine (1 mM) | 23.6 | 362 |
| Butyric acid (0.5 mM) | 31.6 | 476 |
| HMBA (2mM) + Caffeine (1 mM) + butyric acid (0.5 mM) | 46.7 | 553 |

These data indicate that the addition either caffeine (at 1 mM), butyric acid (at 0.5 mM), or HMBA (at 2 mM) had positive effects on both polypeptide production and specific productivity and that the combination of butyric acid, caffeine, and HMBA (at the concentrations mentioned above) had greater positive effects than any of these compounds alone.

EXAMPLE 9

Production of Type II IL-1 Receptor in the Presence of HMBA in a Bioreactor

Nucleic acids encoding a type II IL-1 receptor inserted into a suitable vector were introduced into CHO cells. About 500 thousand cells from a stably transformed line were inoculated into a one liter of serum-free medium in a bioreactor. Cells were grown for two days at 37° C. Thereafter, cells were shifted to 31° C., either without HMBA or in the presence of 2 mM HMBA, and grown for 12 more days. Thereafter, all medium was harvested. The titer of type II IL-1 receptor per milliliter of harvested medium was determined by purification by reverse phase HPLC followed by the measurement of absorbance at 280 nanometers. Data from this experiment are shown in Table 6 as a percentage of the average of the protein titers obtained from the two samples without HMBA rounded to the nearest whole number.

TABLE 6

Effects of 2 mM HMBA on Protein Titer

| Inducer | Relative Titer of type II IL-1 Receptor (percent of average of samples without HMBA) |
|---|---|
| None | 99% |
| None | 101% |
| HMBA (2 mM) | 120% |
| HMBA (2 mM) | 125% |

These data show that bioreactor cultures shifted to 31° C. after an initial 37° C. growth phase produced more type II IL-1 receptor if HMBA was added at the time of temperature shift than if it wasn't. These data further suggest that the invention can be useful for producing a variety of polypeptides in a variety of cell lines and that the mechanics of how the cells are grown, for example, in a shaker flask versus in a bioreactor, are not critical.

EXAMPLE 10

Production of an Antibody Against Murine IL-4 Receptor in CHO Cells

The experiment described below tests the effects of using either sodium butyrate or HMBA as an inducer in still another cell line at various temperatures.

Nucleic acids encoding an antibody against a murine IL-4 receptor inserted into a suitable vector were introduced into CHO cells. About two million cells from a stably transformed line propagated at 37° C. were inoculated into 20 milliliters of medium at the temperatures indicated in FIG. **4** and in the presence or absence of HMBA (2 mM) or sodium butyrate (0.5 mM), as indicated in FIG. **4**. Cells were grown for a maximum of 14 days in a shaker flask. Aliquots were removed at the times indicated in FIG. **4**, and the titer of the antibody (in micrograms per milliliter of harvested medium) was determined by enzyme-linked immunosorbent assay (ELISA), a method well known in the art. See e.g. Reen (1994), Enzyme-Linked Immunosorbent Assay (ELISA), in Basic Protein and Peptide Protocols, Methods Mol. Biol. 32:461–466. The results are shown in FIG. **4**. These data indicate that growth at 31° C. resulted in the production of more antibody for a longer time than growth at either 34° C. or 37° C. when medium was harvested at 7 days or later. These data also indicate that both HMBA and sodium butyrate, individually, enhanced production of the antibody and that HMBA did so to a greater extent than did sodium butyrate at 31° C.

EXAMPLE 11

Production of TNFR:Fc in CHO Cells

Nucleic acids encoding human TNFR:Fc in a suitable vector were introduced into CHO cells. About $3\pm0.5\times10^6$ cells from a stably transformed cell line propagated at 37° C. were introduced into each of three 1 liter bioreactors and cultured at 32.5° C. in an enriched, serum-free medium. Sodium butyrate (0.5 mM) was added to all three cultures, and HMBA (2 mM) was added to two of the cultures ("day 1+HMBA") one day after the shift to 32.5° C. Cells were incubated for a total of 11 days at 32.5° C. Medium was harvested, and protein titer was determined by measuring optical density at 280 nanometers following a prepurifica-

tion using Protein A POROS® Perfusion Chromatography™ (Applied Biosystems, Foster City, Calif., USA). These results are shown in Table 7 as a percentage of the titer obtained from the sample with no HMBA ("day 1") rounded to the nearest whole number.

TABLE 7

Effects of the Timing of Addition of Inducers

| | Relative Titer TNFR:Fc (percent of the day 0 titer) |
|---|---|
| day 1 | 100% |
| day 1 + HMBA | 131% |
| day 1 + HMBA | 110% |

These data indicate that the addition of HMBA increased the titer of TNFR:Fc produced by these cultures when added one day after a temperature shift to 32.5° C. These data, together with the data in previous examples, indicate that addition of HMBA can increase protein titer when it is added at the time of or after a shift to a lower temperature.

The foregoing description of specific embodiments reveals the general nature of the invention so that others can readily modify and/or adapt such embodiments for various applications without departing from the generic concepts presented herein. Any such adaptions or modifications are intended to be embraced within the meaning and range of equivalents of the disclosed embodiments. Phraseology and terminology employed herein are for the purpose of description and not of limitation. All references cited herein are hereby incorporated by reference in their entirety.

What is claimed is:

**1**. A method for producing a polypeptide comprising:

culturing a mammalian cell line in a growth phase followed by a production phase, wherein the production phase occurs at a temperature of less than 37° C.; and

adding to the culture medium during the production phase a xanthine derivative;

wherein the addition of the xanthine derivative increases production of the polypeptide; and

wherein the mammalian cell line is selected from the group consisting of a mammalian cell line that has been genetically engineered to produce the polypeptide and a hybridoma cell line that produces an antibody.

**2**. The method of claim **1**,

wherein the mammalian cell line has been transformed with a recombinant vector encoding the polypeptide, and

wherein the recombinant vector comprises a CMV promoter.

**3**. The method of claim **1**, wherein the xanthine derivative is caffeine at a concentration from about 0.01 millimolar to about 3.0 millimolar.

**4**. The method of claim **1**, wherein the polypeptide is a recombinant fusion polypeptide.

**5**. The method of claim **1**, wherein the antibody polypeptide is a human or humanized antibody.

**6**. The method of claim **1**, wherein the production phase occurs at a temperature from about 29° C. to about 36° C.

**7**. The method of claim **1**, wherein the concentration of each xanthine derivative added to the culture is from about 0.001 millimolar to about 3 millimolar.

**8**. The method of claim **1**, wherein the xanthine derivative is selected from the group consisting of caffeine, 3-isobutyl-1-methylxanthine, theobromine, and pentoxyphylline.

**9**. The method of claim **1**, wherein at least two different xanthine derivatives are added.

**10**. The method of claim **9**, wherein the two different xanthine derivatives are caffeine and 3-isobutyl-1-methylxanthine.

**11**. The method of claim **1**, wherein the mammalian cell line is a CHO cell line.

**12**. The method of claim **11**, wherein the CHO cell line is exposed to the xanthine derivative or derivatives for at least about 5 days.

**13**. The method of claim **1**, wherein the medium used during the production phase is serum free.

**14**. The method of claim **1**, further comprising collecting the polypeptide from the medium.

**15**. The method of claim **14**, further comprising formulating the polypeptide.

**16**. The method of claim **1**, further comprising multiple additions of the xanthine derivative.

**17**. The method of claim **1**, wherein the medium further comprises a hybrid polar compound.

**18**. The method of claim **17**, wherein the medium further comprises an alkanoic acid.

**19**. The method of claim **1**, wherein the medium further comprises an alkanoic acid.

**20**. The method of claim **17**, wherein the hybrid polar compound is hexamethylene bisacetamide and the xanthine derivative is caffeine.

**21**. The method of claim **20**,

wherein the medium further comprises a salt of butyric acid at a concentration from about 0.1 millimolar to about 2 millimolar,

wherein hexamethylene bisacetamide is at a concentration from about 0.1 millimolar to about 5 millimolar, and

wherein caffeine is at a concentration from about 0.01 millimolar to about 5 millimolar.

**22**. The method of claim **17**, wherein the mammalian cell line is cultured at a temperature from about 29° C. to about 36° C.

**23**. The method of claim **22**, wherein the mammalian cell line is cultured at a temperature from about 30° C. to about 33° C.

**24**. The method of claim **22**,

wherein the mammalian cell line is cultured in the growth phase at a first temperature from about 35° C. to about 38° C. before it is shifted to the production phase at a second temperature from about 29° C. to about 36° C. and

wherein the hybrid polar compound and the xanthine are added after the shift from the first temperature to the second temperature.

**25**. The method of claim **18**, wherein the mammalian cell line is cultured at a temperature from about 29° C. to about 36° C.

**26**. A method for producing a recombinant polypeptide comprising:

culturing a CHO cell line that has been genetically engineered to produce the recombinant polypeptide; and

adding to the culture medium at least one xanthine derivative selected from the group consisting of theobromine and caffeine,

wherein the addition of the xanthine derivative increases the production of the recombinant polypeptide.

**27**. The method of claim **26**,

wherein the CHO cell line has been transformed with a recombinant vector encoding the recombinant polypeptide and

wherein the recombinant vector comprises a CMV promoter.

**28**. The method of claim **26**, wherein the recombinant polypeptide is a fusion polypeptide.

**29**. The method of claim **26**, wherein the recombinant polypeptide is a human or humanized antibody.

**30**. The method of claim **26**, wherein the concentration of each xanthine derivative added to the culture medium is from about 0.001 millimolar to about 3 millimolar.

**31**. The method of claim **26**, wherein the xanthine derivative is caffeine.

**32**. The method of claim **26**, further comprising collecting the recombinant polypeptide from the medium.

**33**. The method of claim **32**, further comprising formulating the recombinant polypeptide.

**34**. The method of claim **26**, further comprising multiple additions of the xanthine derivative.

**35**. The method of claim **26**, wherein the CHO cell line is cultured at a temperature from about 29° C. to about 36° C.

**36**. The method of claim **35**, wherein the CHO cell line is cultured at a temperature from about 30° C. to about 33° C.

**37**. The method of claim **35**,

wherein the CHO cell line is cultured at a first temperature from about 35° C. to about 38° C. before it is shifted to a second temperature from about 29° C. to about 36° C. and

wherein the xanthine derivative is added after the shift from the first temperature to the second temperature.

**38**. A culture comprising a CHO cell genetically engineered to produce a polypeptide, a production medium, and a xanthine derivative selected from the group consisting of caffeine, theobromine, and pentoxyphylline.

**39**. The culture of claim **38**, wherein the concentration of each xanthine derivative present is from about 0.01 millimolar to about 3 millimolar.

**40**. The culture of claim **38**, wherein the production medium is serum-free.

**41**. The culture of claim **38**, wherein the culture comprises at least two xanthine derivatives.

**42**. A method for producing a recombinant polypeptide comprising

culturing a mammalian cell line at a temperature from about 29° C. to about 36° C. and

adding a hybrid polar compound to the culture medium,

wherein the mammalian cell line has been genetically engineered to produce the recombinant polypeptide and

wherein the addition of the hybrid polar compound increases the production of the recombinant polypeptide.

**43**. The method of claim **42**, wherein the hybrid polar compound is hexamethylene bisacetamide at a concentration from about 0.1 millimolar to about 20 millimolar.

**44**. The method of claim **42**,

wherein the mammalian cell line is cultured at a first temperature from about 35° C. to about 38° C. before it is shifted to a second temperature from about 29° C. to about 36° C. and

wherein the hybrid polar compound is added is added after the shift from the first temperature to the second temperature.

**45**. The method of claim **42**, wherein the medium further comprises an alkanoic acid at a concentration from about 0.05 millimolar to about 10 millimolar.

**46**. The method of claim **43**, wherein the hexamethylene bisacetamide in the medium is present at a concentration from about 0.1 millimolar to about 5 millimolar.

**47**. The method of claim **42**, wherein the mammalian cell line is cultured at a temperature from about 30° C. to about 33° C.

**48**. The method of claim **45**, wherein

the alkanoic acid is a salt of butyric acid, and

the concentration of the salt of butyric acid is from about 0.1 millimolar to about 2 millimolar.

**49**. The method of claim **45**, wherein

the hybrid polar compound is hexamethylene bisacetamide at a concentration from about 0.1 millimolar to about 5 millimolar, and

the alkanoic acid is a salt of butyric acid at a concentration from about 0.1 millimolar to about 2 millimolar.

**50**. The method of claim **42**, wherein the medium is serum free.

**51**. The method of claim **42**, wherein the medium further comprises a xanthine derivative.

**52**. The method of claim **51**, wherein the xanthine derivative is caffeine.

**53**. The method of claim **51**, wherein the medium further comprises a salt of butyric acid.

**54**. The method of claim **53**, wherein the xanthine derivative is present at a concentration from about 0.01 millimolar to about 3 millimolar and the salt of butyric acid is present at a concentration from about 0.1 millimolar to about 2 millimolar.

**55**. The method of claim **42**, wherein the recombinant polypeptide is a secreted polypeptide.

**56**. The method of claim **55**, further comprising recovering the recombinant polypeptide from the medium.

**57**. The method of claim **42**, wherein the mammalian cell line is a CHO cell line.

**58**. A method for producing a polypeptide comprising

culturing a mammalian cell line that can express the polypeptide in a growth phase at a first temperature from about 35° C. to about 38° C., and then

culturing the mammalian cell line in a production phase at a second temperature from about 30° C. to 34° C. in a medium comprising a hybrid polar compound.

**59**. The method of claim **58**, wherein the polypeptide is a recombinant polypeptide or an antibody.

**60**. The method of claim **58**, wherein the hybrid polar compound is hexamethylene bisacetamide.

**61**. The method of claim **58**, wherein the medium further comprises an alkanoic acid.

**62**. The method of claim **61**, wherein the hybrid polar compound is hexamethylene bisacetamide and the alkanoic acid is a salt of butyric acid.

**63**. The method of claim **58**, wherein the medium is serum free.

**64**. The method of claim **58**, wherein the hybrid polar compound is added after the shift from the first temperature to the second temperature.

**65**. The method of claim **58**, wherein

the medium further comprises a xanthine derivative at a concentration from about 0.001 millimolar to about 5.0 millimolar and an alkanoic acid at a concentration from about 0.05 millimolar to about 10.0 millimolar and

the hybrid polar compound is hexamethylene bisacetamide at a concentration from about 0.1 millimolar to about 5 millimolar.

**66**. The method of claim **58**, wherein the mammalian cell line is a hybridoma cell line or a CHO cell line.

**67**. A method for producing a polypeptide comprising culturing mammalian cells that can produce the polypeptide in a medium comprising between about 0.1 millimolar and about 5 millimolar hexamethylene bisacetamide, from about 0.1 millimolar to about 2 millimolar butyric acid, and from about 0.1 millimolar to about 4 millimolar caffeine at a temperature from about 30° C. to about 36° C.

**68**. The method of claim **67**, wherein the polypeptide is selected from the group consisting of RANK:Fc, type II interleukin-1 receptor, TNFR:Fc, CD40 ligand, TRAIL, flt3-ligand, IL-4 receptor, G-CSF, erythropoietin, an antibody, and substantially similar polypeptides.

**69**. A cell culture comprising a CHO cell line that has been genetically engineered to produce a polypeptide and a production medium comprising hexamethylene bisacetamide, wherein the culture is incubated at a temperature from about 30° C. to 36° C. for at least about 5 days.

* * * * *

UNITED STATES PATENT AND TRADEMARK OFFICE

# CERTIFICATE OF CORRECTION

PATENT NO. : 6,872,549 B2
APPLICATION NO. : 10/400334
DATED : March 29, 2005
INVENTOR(S) : Kirk P. Van Ness et al.



It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Column 14, line 63, "arc" should read -- are --.

Column 23, line 22, "(FLAGS" should read -- (FLAG®) --.

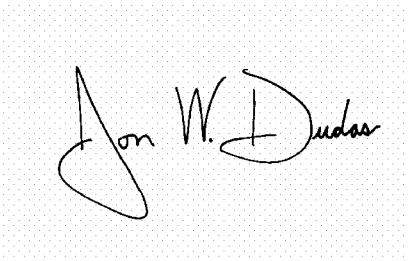
Column 29, line 36, "tier" should read -- titer --.

Column 31, line 13, remove the second occurrence of "of".

Column 31, line 38, "addition either" should read -- addition of either --.

Signed and Sealed this

Twenty-sixth Day of December, 2006



JON W. DUDAS
*Director of the United States Patent and Trademark Office*

# EXHIBIT 4

US006924124B1

(12) **United States Patent**
**Singh**

(10) **Patent No.: US 6,924,124 B1**
(45) **Date of Patent: Aug. 2, 2005**

(54) **FEEDING STRATEGIES FOR CELL CULTURE**

(75) Inventor: **Pankaj Singh**, Seattle, WA (US)

(73) Assignee: **Immunex Corporation**, Seattle, WA (US)

( * ) Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 0 days.

(21) Appl. No.: **10/227,154**

(22) Filed: **Aug. 23, 2002**

(51) **Int. Cl.**[7] ..... **C12P 21/04**; C12P 21/06

(52) **U.S. Cl.** ..... **435/70.1**; 435/69.1; 435/71.1; 435/70.3

(58) **Field of Search** ..... 435/69.1, 70.3, 435/253, 320.1, 252, 70.1, 71.1, 803, 325, 435/366, 404; 530/351, 410, 350; 514/12

(56) **References Cited**

U.S. PATENT DOCUMENTS

| | | | | |
|---|---|---|---|---|
| 4,931,543 | A * | 6/1990 | Halenbeck et al. | 530/351 |
| 5,407,810 | A * | 4/1995 | Builder et al. | 435/69.1 |
| 5,705,364 | A * | 1/1998 | Etcheverry et al. | 435/70.3 |
| 5,789,199 | A * | 8/1998 | Joly et al. | 435/69.1 |
| 6,103,529 | A * | 8/2000 | Price et al. | 435/404 |

OTHER PUBLICATIONS

Liu, Yung-Chuan, Cultivation of Recombinant *E. coli* to Achieve High Cell Density with High Level of Pnencilin G Acylase Activity., Dec. 9, 1999, Proc. Natl. Sci Coun. vol. 24, No. 4. 2000. pp. 156-160.*

deZengotita et al., "Phosphate Feeding Improves High-Cell-Concentration NS0 Myeloma Culture Performance for Monoclonal Antibody Production,"*Biotechnology and Bioengineering 69*(5):566-576, 2000.

Jo et al., "Step-Fortifications of Nutrients in Mammalian Cell Culture,"*Biotechnology and Bioengineering 42*:1218-1228, 1993.

Sato et al, "Stimulation of monoclonal antibody production by human-human hybridoma cells with an elevated concentration of potassium or sodium phosphate in serum-free medium,"*Cytotechnology 2*:63-67, 1989.

* cited by examiner

*Primary Examiner*—Robert A. Wax
*Assistant Examiner*—Robert Mondesi
(74) *Attorney, Agent, or Firm*—Kathleen Fowler

(57) **ABSTRACT**

The invention is in the field of cell culture, particularly recombinant cell culture. More specifically, the invention relates to methods of fed batch CHO cell culture.

**18 Claims, 2 Drawing Sheets**

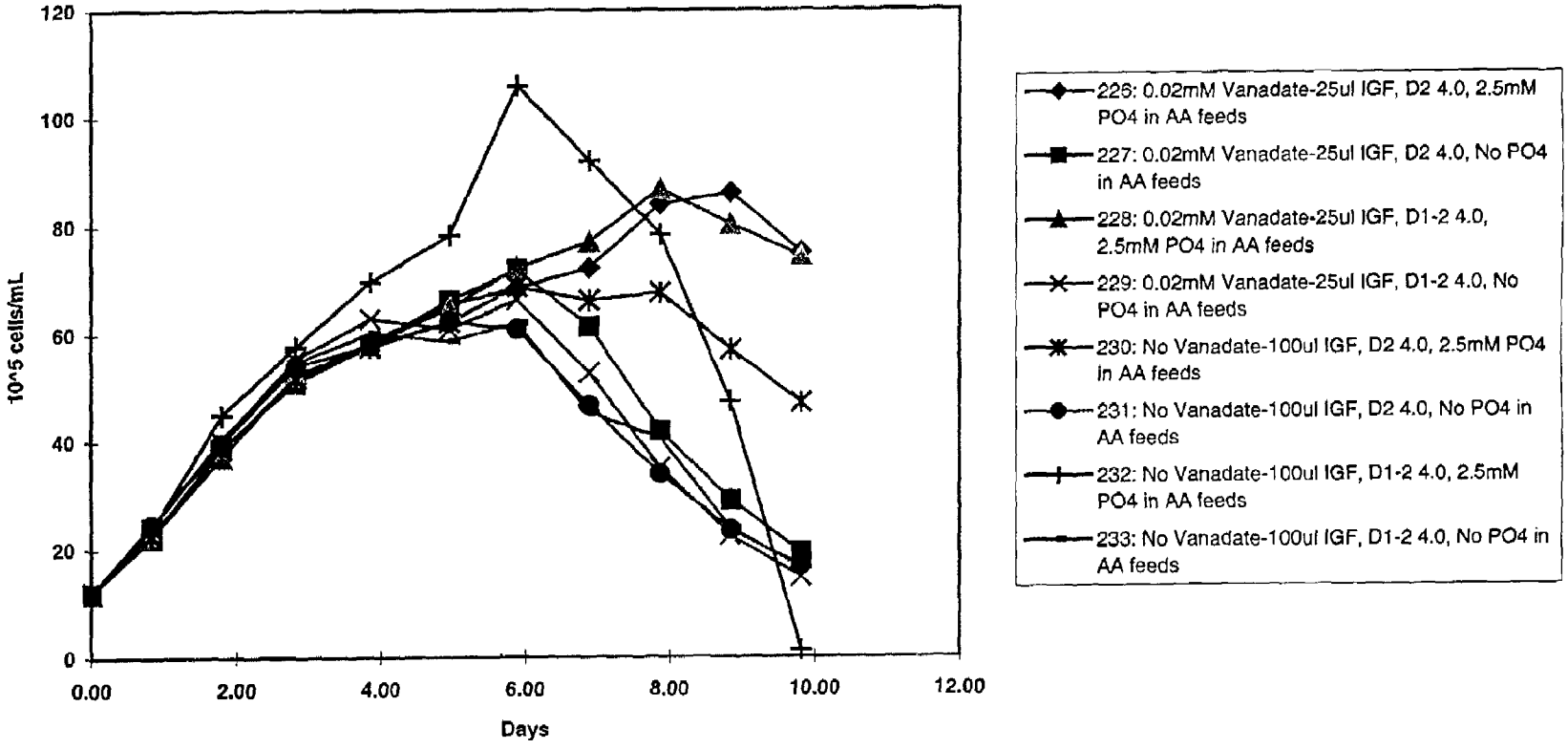

120
100
80
60
40
20
0
10^5 cells/mL
0.00
2.00
4.00
6.00
8.00
10.00
12.00
Days
226: 0.02mM Vanadate-25ul IGF, D2 4.0, 2.5mM PO4 in AA feeds
227: 0.02mM Vanadate-25ul IGF, D2 4.0, No PO4 in AA feeds
228: 0.02mM Vanadate-25ul IGF, D1-2 4.0, 2.5mM PO4 in AA feeds
229: 0.02mM Vanadate-25ul IGF, D1-2 4.0, No PO4 in AA feeds
230: No Vanadate-100ul IGF, D2 4.0, 2.5mM PO4 in AA feeds
231: No Vanadate-100ul IGF, D2 4.0, No PO4 in AA feeds
232: No Vanadate-100ul IGF, D1-2 4.0, 2.5mM PO4 in AA feeds
233: No Vanadate-100ul IGF, D1-2 4.0, No PO4 in AA feeds

FIGURE 1

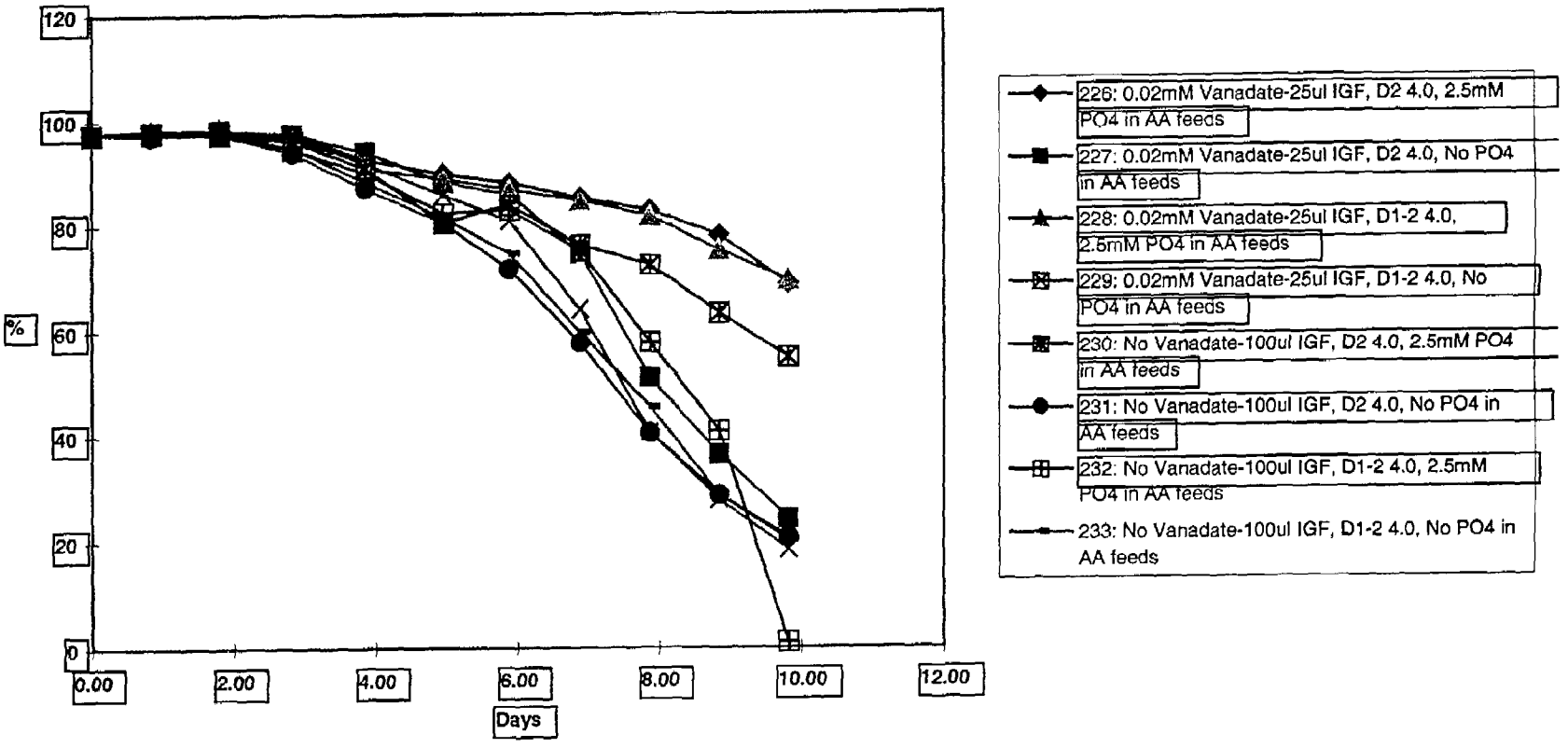

120
100
80
60
40
20
0
%
0.00
2.00
4.00
6.00
8.00
10.00
12.00
Days
226: 0.02mM Vanadate-25ul IGF, D2 4.0, 2.5mM PO4 in AA feeds
227: 0.02mM Vanadate-25ul IGF, D2 4.0, No PO4 in AA feeds
228: 0.02mM Vanadate-25ul IGF, D1-2 4.0, 2.5mM PO4 in AA feeds
229: 0.02mM Vanadate-25ul IGF, D1-2 4.0, No PO4 in AA feeds
230: No Vanadate-100ul IGF, D2 4.0, 2.5mM PO4 in AA feeds
231: No Vanadate-100ul IGF, D2 4.0, No PO4 in AA feeds
232: No Vanadate-100ul IGF, D1-2 4.0, 2.5mM PO4 in AA feeds
233: No Vanadate-100ul IGF, D1-2 4.0, No PO4 in AA feeds

FIGURE 2

# FEEDING STRATEGIES FOR CELL CULTURE

## FIELD OF THE INVENTION

The invention is in the field of cell culture, particularly recombinant cell culture. More specifically, the invention relates to methods of fed batch CHO cell culture.

## BACKGROUND

One goal of recombinant protein production is the optimization of culture conditions so as to obtain the greatest possible productivity. Even incremental increases in productivity can be economically significant.

CHO (Chinese hamster ovary) cell lines are often used for recombinant protein production because they grow well in either adherent or suspension culture, and efficiently produce many proteins. Further, CHO cells and recombinant proteins expressed in them have been extensively characterized and have been approved for use in clinical manufacturing by regulatory agencies.

Some of the methods to increase productivity in CHO cell culture include using enriched medium, monitoring and altering osmolarity during production, decreasing temperatures during specific phases of a cell culture, and/or the addition of sodium butyrate to induce expression during the production phase (see, for example, U.S. Pat. No. 5,705,364 to Etcheverry et al.). In addition, when CHO cells are grown in batch culture, periodic feeding of the cells with essential nutrients will also increase production (see, for example, U.S. Pat. No. 5,672,502 to Birch et al.).

However, there remains a need in the art to continually improve yields of recombinant protein from each cell culture run.

## SUMMARY OF THE INVENTION

The invention relates to improved and optimized methods of producing recombinant proteins in CHO cells. In particular, the invention provides a method of producing a recombinant protein, the method comprising culturing a CHO cell culture genetically engineered to produce the protein in a tissue culture medium, and adding a feed solution to the cell culture, wherein the feed solution comprises an effective amount of a phosphate compound. Generally, it has been found that the phosphate should be added to achieve an increase in the final cell culture concentration of around 1 millimolar to about 10 millimolar phosphate. The phosphate compound can be selected from the group consisting of sodium phosphate, potassium phosphate, phosphoric acid, and other salts of phosphoric acid.

Optionally, the feed solution additionally comprises one or more amino acids. The invention finds particular use when the cells are under inducing conditions when the feed solution is added. The feed solution is added repeatedly, such as, for example, about every two days for 4 to 10 days. The methods of the invention result in increased production of the recombinant protein by the CHO cell culture as compared to the CHO cell culture in the absence of added feed solution. The methods of the invention are particularly useful for large scale culturing of CHO cell cultures.

## BRIEF DESCRIPTION OF THE FIGURES

FIG. **1** is a graph of the viable cell density (VCD) over time for the following eight conditions: tank **226** was 0.02 mM Vanadate-25 ul IGF, D2 4.0, 2.5 mM PO4 in feeds (diamonds); tank **227** was 0.02 mM Vanadate-25 ul IGF, D2 4.0, No PO4 in feeds (squares); tank **228** was 0.02 mM Vanadate-25 ul IGF, D1-2 4.0, 2.5 mM PO4 (triangles); tank **229** was 0.02 mM Vanadate-25 ul IGF, D1-2 4.0, No PO4 in feeds (X's); tank **230** was No Vanadate-100 ul IGF, D2 4.0, 2.5 mM PO4 in feeds (stars); tank **231** was No Vanadate-100 ul IGF, D2 4.0, No PO4 in feeds (circles); tank **232** was No Vanadate-100 ul IGF, D1-2 4.0, 2.5 mM PO4 in feeds (crosses); tank **233** was No Vanadate-100 ul IGF, D1-2 4.0, No PO4 in feeds (narrow rectangles).



FIG. **2** is a graph of the percent viability over time, for each of the 8 different tanks. Conditions and symbols are the same as for the previous figure.

## DETAILED DESCRIPTION OF THE INVENTION

During CHO batch phase culture of recombinant cells, nutrients can become limiting, leading to a reduction in cell performance (as measured by cell viability, viable cell density, and recombinant protein production). To overcome these effects, batch cultures can be fed with a concentrated solution of medium and/or amino acids. This process is known as fed batch culture. During experiments with CHO fed batch culture, it was noticed that the acid used to buffer solutions for the feeds had an effect on performance of the cell culture. In particular, it was noticed that cultures fed amino acids buffered with phosphoric acid performed better than cultures fed amino acids buffered with hydrochloric acid. Additional experimentation determined that this effect was not due to the buffering action of the acid, but rather to the presence or absence of phosphate. Specifically, when CHO cultures were fed amino acids buffered with hydrochloric acid, with or without the addition of phosphate, the cultures receiving phosphate had enhanced performance.

Thus, the invention provides improved methods of producing recombinant proteins using batch culture in CHO cells. In particular, the invention provides a method of producing a recombinant protein, the method comprising culturing in batch culture in a tissue culture medium a CHO cell culture genetically engineered to produce the protein, and adding a feed solution to the cell culture, wherein the feed solution comprises an effective amount of a phosphate compound.

The amount and timing of addition of phosphate compound to the cell culture will vary slightly by cell line, and can be optimized by those skilled in the art. Generally, for most fed batch processes, it has been found that the phosphate compound should be added so as to achieve an increase in the cell culture concentration of around 0.1 millimolar to about 10 millimolar phosphate just after addition of the feed. Feed solutions can be added repeatedly. More frequent feeds will call for the addition of lower amounts of phosphate compound each time; conversely, less frequent feeds will call for the addition of higher amounts of phosphate compound. However, very high concentrations of phosphate in the cell culture should be avoided as such can be toxic to CHO cells. In illustrative embodiments described below, a feed solution containing a phosphate compound is added about every two days in an amount to result in a concentration of phosphate in the cell culture of about 1.5 to about 3.5 mM, preferably about 2.5 mM phosphate.

The phosphate compound can be added in any non-conjugated form that is not toxic to the cell. For example, the phosphate compound can be selected from the group consisting of sodium phosphate, potassium phosphate, phos-

phoric acid, and other salts of phosphoric acid. The phosphate compound can be added along with other nutrients in the feed. Other nutrients can include, but are not limited to, any combination of the following: L-Glutamine, L-Asparagine, L-Proline, L-Methionine, L-Isoleucine, L-Leucine, L-Phenylalanine, L-Tryptophan, L-Lysine, L-Histidine, L-Arginine, L-Serine, L-Glycine, L-Threonine, L-Valine, L-Cystine, L-Tyrosine, IGF-1, insulin, hydrocortisone, sodium bicarbonate, dichloroacetate, acids, bases, glucose, other carbohydrates, peptones, hydrosylates, and vitamins. For example, the feed can contain a concentrated medium solution with a phosphate compound, and/or various additions of amino acids with a phosphate compound. In a non-limiting, illustrative embodiment below, at least one of the feeds contain a concentrated solution of 17 amino acids. The feeds can be different in composition on different days, or the same. An effective amount of phosphate compound will result in increased production of the recombinant protein by the cell culture as compared to the CHO cell culture in the which has been fed a feed solution that does not contain the phosphate compound.

The proteins can be produced recombinantly in CHO (Chinese hamster ovary) cells and are preferably secreted by CHO cells adapted to grow in cell culture. Preferably, the host cells are homogenous CHO cell lines. Such host cells are available from a number of depositaries and laboratories, such as the ATCC. The dihydrofolate reductase (DHFR)-deficient mutant cell line (Urlaub et al., 1980, Proc Natl Acad Sci USA 77:4216–4220), DXB11 and DG-44, are the CHO host cell lines of choice because the efficient DHFR selectable and amplifiable gene expression system allows high level recombinant protein expression in these cells (Kaufman R. J., 1990, Meth Enzymol 185:527–566). In addition, these cells are easy to manipulate as adherent or suspension cultures and exhibit relatively good genetic stability. In addition, new animal cell lines can be established using methods well known by those skilled in the art (e.g., by transformation, viral infection, and/or selection, etc.).

By in vitro cell culture is meant the growth and propagation of cells outside of a multicellular organism or tissue. Typically, in vitro cell culture is performed under sterile, controlled temperature and atmospheric conditions in tissue culture plates (e.g., 10 cm plates, 96 well plates, etc.), or other adherent culture (e.g., on microcarrier beads) or in suspension culture and/or in roller bottles. Cultures can be grown in shake flasks, small scale bioreactors, and/or large-scale bioreactors. A bioreactor is a device used to culture animal cells in which environmental conditions such as temperature, atmosphere, agitation, and/or pH can be monitored and adjusted. A number of companies (e.g., ABS Inc., Wilmington, Del.; Cell Trends, Inc., Middletown, Md.) as well as university and/or government-sponsored organizations (e.g., The Cell Culture Center, Minneapolis, Minn.) offer cell culture services on a contract basis.

Further, the methods and cell cultures of the invention (adherent or non-adherent and growing or growth arrested), can be small scale cultures, such as for example in 100 ml containers having about 30 ml of media, 250 ml containers having about 80 to 90 ml of media, 250 ml containers having about 150 to 200 ml of media. Alternatively, the cultures can be large scale such as for example 1000 ml containers having about 300 to 1000 ml of media, 3000 ml containers having about 500 to 3000 ml of media, 8000 ml containers having about 2000 to about 8000 ml of media, and 15000 ml containers having about 4000 ml to about 15000 ml of media. Both small scale and large scale culturing can be performed in bioreactors. In preferred embodiments, the size of the culture is at least about 100 liters, more preferably at least about 1000 liters, still more preferably at least about 5000 liters, even more preferably at least about 7000 liters.

Various tissue culture media, including serum-free and/or defined culture media, are commercially available for cell culture. Tissue culture medium is defined, for purposes of the invention, as a medium suitable for growth of animal cells, and preferably mammalian cells, in in vitro cell culture. Typically, tissue culture media contains a buffer, salts, energy source, amino acids, vitamins and trace essential elements. Any medium capable of supporting growth of the appropriate eukaryotic cell in culture can be used; as shown below by way of example, variations in a serum-free medium composition did not affect the superior results obtained when phosphate was fed to the cell culture. Tissue culture media suitable for use in the invention are commercially available from, e.g., ATCC (Manassas, Va.). For example, any one or combination of the following media can be used: RPMI-1640 Medium, Dulbecco's Modified Eagle's Medium, Minimum Essential Medium Eagle, F-12K Medium, Iscove's Modified Dulbecco's Medium. When defined medium that is serum-free and/or peptone-free is used, the medium is usually highly enriched for amino acids and trace elements (see, for example, U.S. Pat. No. 5,122,469 to Mather et al., and U.S. Pat. No. 5,633,162 to Keen et al.).

In the methods and compositions of the invention, cells can be grown in serum-free, protein-free, growth factor-free, and/or peptone-free media. The term "serum-free" as applied to media includes any mammalian cell culture medium that does not contain serum, such as fetal bovine serum. The term "insulin-free" as applied to media includes any medium to which no exogenous insulin has been added. By exogenous is meant, in this context, other than that produced by the culturing of the cells themselves. The term "IGF-1-free" as applied to media includes any medium to which no exogenous Insulin-like growth factor-1 (IGF-1) or analog (such as, for example, LongR$^3$-IGF-1, see below) has been added. The term "growth-factor free" as applied to media includes any medium to which no exogenous growth factor (e.g., insulin, IGF-1) has been added. The term "protein-free" as applied to media includes medium free from exogenously added protein, such as, for example, transferrin and the protein growth factors IGF-1 and insulin. Protein-free media may or may not have peptones. The term "peptone-free" as applied to media includes any medium to which no exogenous protein hydrolysates have been added such as, for example, animal and/or plant protein hydrolysates. Peptone-free media has the advantages of lower lot to lot variability and fewer filtration problems than media containing plant or animal hydrolysates. Chemically defined media are media in which every component is defined and obtained from a pure source, preferably a non-animal source.

Preferably, the medium used is serum-free, or essentially serum-free. By "essentially serum-free" is meant that less than about 2% serum is present, more preferably less than about 1% serum is present, still more preferably less than about 0.5% serum is present, yet still more preferably less than about 0.1% serum is present.

Batch culture is well known in the art, as are methods of fed batch culture (see U.S. Pat. No. 5,672,502). Cells are cultured in a fixed volume, and supplementary nutrients are added. The methods of the invention can be used in combination with other types of culture. For example, cell cultures can be serial subcultured in larger and larger vol-

umes of culture medium to as to maintain the cells in exponential phase, and then converted to a batch culture system when a desired volume or cell density is achieved. Then, the batch cell culture can be fed using the methods of the invention. For example, a CHO cell culture can be grown and progressively transferred from a small scale culture to a large scale culture, and then seeded at a desired cell density into a batch cell culture. Once in the batch cell culture, the cells can be fed using the methods of the invention. CHO cells can be maintained in batch culture for as long as recombinant protein production occurs. Preferably, the batch culture is maintained in a production phase for about 2 to about 16 days, more preferably for about 6 to about 12 days.

Further, the methods of the invention can be used in combination with known or yet to be discovered methods of inducing the production of recombinant proteins. By "inducing conditions" is meant a technique to increase the relative production per cell of a desired recombinant protein. Often, other cell processes (such as growth and division) are inhibited so as to direct most of the cells' energy into recombinant protein production. Such techniques include cold temperature shift, and additions of chemicals such as sodium butyrate (as described in U.S. Pat. No. 5,705,364 to Etcheverry et al., incorporated herein by reference), DMSO, DMF, DMA, TNF-alpha, phorbol 12-myristate 13-acetate, PMA, propionate, forskolin, dibutyryl cAMP, 2-aminopurine, adenine, adenosine, okadaic acid, and combinations of any of these techniques, to name just a few examples, as well as any yet to be described and/or discovered induction techniques. Typically, a batch culture of cells at high density is induced to produce the recombinant protein.

The invention can be used in the culture of cells that produce just about any protein, especially recombinant proteins. Examples of useful expression vectors that can be used to produce proteins are disclosed in WO 01/27299, and the pDC409 vector described in McMahan et al., 1991, Embo J. 10:2821. A protein is generally understood to be a polypeptide of at least about 10 amino acids, more preferably at least about 25 amino acids, even more preferably at least about 75 amino acids, and most preferably at least about 100 amino acids.

Generally, the methods of the invention are useful for the production of recombinant proteins. Recombinant proteins are proteins produced by the process of genetic engineering. The term "genetic engineering" refers to a recombinant DNA or RNA method used to create a host cell that expresses a gene at elevated levels, at lowered levels, or a mutant form of the gene. In other words, the cell has been transfected, transformed or transduced with a recombinant polynucleotide molecule, and thereby altered so as to cause the cell to alter expression of a desired protein. Methods and vectors for genetically engineering cells and/or cell lines to express a protein of interest are well known to those skilled in the art; for example, various techniques are illustrated in *Current Protocols in Molecular Biology*, Ausubel et al., eds. (Wiley & Sons, New York, 1988, and quarterly updates) and Sambrook et al., *Molecular Cloning: A Laboratory Manual* (Cold Spring Laboratory Press, 1989). Genetic engineering techniques include but are not limited to expression vectors, targeted homologous recombination and gene activation (see, for example, U.S. Pat. No. 5,272,071 to Chappel) and trans activation by engineered transcription factors (see, for example, Segal et al., 1999, Proc. Natl. Acad. Sci. USA 96(6):2758–63). Preferrably, the proteins are expressed under the control of a heterologous control element such as, for example, a promoter that does not in nature direct the production of that protein. For example, the promoter can be a strong viral promoter (e.g., CMV, SV40) that directs the expression of a mammalian protein. The host cell may or may not normally produce the protein. For example, the host cell can be a CHO cell that has been genetically engineered to produce a human protein. Alternatively, the host cell can be a human cell that has been genetically engineered to produce increased levels of a human protein normally present only at very low levels (e.g., by replacing the endogenous promoter with a strong viral promoter).

Particularly preferred proteins for expression are protein-based therapeutics, also known as biologics. Preferably, the proteins are secreted as extracellular products. Proteins that can be produced using the invention include but are not limited to Flt3 ligand, CD40 ligand, erythropoeitin, thrombopoeitin, calcitonin, Fas ligand, ligand for receptor activator of NF-kappa B (RANKL), TNF-related apoptosis-inducing ligand (TRAIL), ORK/Tek, thymic stroma-derived lymphopoietin, granulocyte colony stimulating factor, granulocyte-macrophage colony stimulating factor, mast cell growth factor, stem cell growth factor, epidermal growth factor, RANTES, growth hormone, insulin, insulinotropin, insulin-like growth factors, parathyroid hormone, interferons, nerve growth factors, glucagon, interleukins 1 through 18, colony stimulating factors, lymphotoxin-β, tumor necrosis factor, leukemia inhibitory factor, oncostatin-M, and various ligands for cell surface molecules Elk and Hek (such as the ligands for eph-related kinases, or LERKS). Descriptions of proteins that can be produced according to the invention may be found in, for example, *Human Cytokines: Handbook for Basic and Clinical Research*, Vol. II (Aggarwal and Gutterman, eds. Blackwell Sciences, Cambridge Mass., 1998); *Growth Factors: A Practical Approach* (McKay and Leigh, eds., Oxford University Press Inc., New York, 1993) and *The Cytokine Handbook* (AW Thompson, ed.; Academic Press, San Diego Calif.; 1991).

Production of the receptors for any of the aforementioned proteins can also be improved using the invention, including the receptors for both forms of tumor necrosis factor receptor (referred to as p55 and p75), Interleukin-1 receptors (type 1 and 2), Interleukin-4 receptor, Interleukin-15 receptor, Interleukin-17 receptor, Interleukin-18 receptor, granulocyte-macrophage colony stimulating factor receptor, granulocyte colony stimulating factor receptor, receptors for oncostatin-M and leukemia inhibitory factor, receptor activator of NF-kappa B (RANK), receptors for TRAIL, and receptors that comprise death domains, such as Fas or Apoptosis-Inducing Receptor (AIR). A particularly preferred receptor is a soluble form of the IL-1 receptor type II; such proteins are described in U.S. Pat. No. 5,767,064, incorporated herein by reference in its entirety.

Other proteins that can be produced using the invention include cluster of differentiation antigens (referred to as CD proteins), for example, those disclosed in *Leukocyte Typing VI* (*Proceedings of the VIth International Workshop and Conference*; Kishimoto, Kikutani et al., eds.; Kobe, Japan, 1996), or CD molecules disclosed in subsequent workshops. Examples of such molecules include CD27, CD30, CD39, CD40; and ligands thereto (CD27 ligand, CD30 ligand and CD40 ligand). Several of these are members of the TNF receptor family, which also includes 41BB and OX40; the ligands are often members of the TNF family (as are 41BB ligand and OX40 ligand); accordingly, members of the TNF and TNFR families can also be produced using the present invention.

Proteins that are enzymatically active can also be produced according to the instant invention. Examples include metalloproteinase-disintegrin family members, various

kinases, glucocerebrosidase, alpha-galactosidase A, superoxide dismutase, tissue plasminogen activator, Factor VIII, Factor IX, apolipoprotein E, apolipoprotein A-I, globins, an IL-2 antagonist, alpha-1 antitrypsin, TNF-alpha Converting Enzyme, and numerous other enzymes. Ligands for enzymatically active proteins can also be produced by applying the instant invention.

The inventive compositions and methods are also useful for production of other types of recombinant proteins, including immunoglobulin molecules or portions thereof, and chimeric antibodies (i.e., an antibody having a human constant region couples to a murine antigen binding region) or fragments thereof. Numerous techniques are known by which DNA encoding immunoglobulin molecules can be manipulated to yield DNAs capable of encoding recombinant proteins such as single chain antibodies, antibodies with enhanced affinity, or other antibody-based polypeptides (see, for example, Larrick et al., 1989, Biotechnology 7:934–938; Reichmann et al., 1988, Nature 332:323–327; Roberts et al., 1987, Nature 328:731–734; Verhoeyen et al., 1988, Science 239:1534–1536; Chaudhary et al., 1989, Nature 339:394–397). Recombinant cells producing fully human antibodies (such as are prepared using transgenic animals, and optionally further modified in vitro), as well as humanized antibodies, can also be used in the invention. The term humanized antibody also encompasses single chain antibodies. See, e.g., Cabilly et al., U.S. Pat. No. 4,816,567; Cabilly et al., European Patent No. 0,125,023 B1; Boss et al., U.S. Pat. No. 4,816,397; Boss et al., European Patent No. 0,120,694 B1; Neuberger, M. S. et al., WO 86/01533; Neuberger, M. S. et al., European Patent No. 0,194,276 B1; Winter, U.S. Pat. No. 5,225,539; Winter, European Patent No. 0,239,400 B1; Queen et al., European Patent No. 0 451 216 B1; and Padlan, E. A. et al., EP 0 519 596 A1. For example, the invention can be used in the production of human and/or humanized antibodies that immunospecifically recognize specific cellular targets, e.g., any of the aforementioned proteins, the human EGF receptor, the her-2/neu antigen, the CEA antigen, Prostate Specific Membrane Antigen (PSMA), CD5, CD11a, CD18, NGF, CD20, CD45, CD52, Ep-cam, other cancer cell surface molecules, TNF-alpha, TGF-b1, VEGF, other cytokines, alpha 4 beta 7 integrin, IgEs, viral proteins (for example, cytomegalovirus), etc., to name just a few.

Various fusion proteins can also be produced using the invention. A fusion protein is a protein, or domain or a protein (e.g. a soluble extracellular domain) fused to a heterologous protein or peptide. Examples of such fusion proteins include proteins expressed as a fusion with a portion of an immunoglobulin molecule, proteins expressed as fusion proteins with a zipper moiety, and novel polyfunctional proteins such as a fusion proteins of a cytokine and a growth factor (i.e., GM-CSF and IL-3, MGF and IL-3). WO 93/08207 and WO 96/40918 describe the preparation of various soluble oligomeric forms of a molecule referred to as CD40L, including an immunoglobulin fusion protein and a zipper fusion protein, respectively; the techniques discussed therein are applicable to other proteins. Another fusion protein is a recombinant TNFR:Fc, also known as "entanercept." Entanercept is a dimer of two molecules of the extracellular portion of the p75 TNF alpha receptor, each molecule consisting of a 235 amino acid TNFR-derived polypeptide that is fused to a 232 amino acid Fc portion of human IgG1. In fact, any of the previously described molecules can be expressed as a fusion protein including but not limited to the extracellular domain of a cellular receptor molecule, an enzyme, a hormone, a cytokine, a portion of an immunoglobulin molecule, a zipper domain, and an epitope.

After culturing using the methods of the invention, the resulting expressed protein can then be collected. In addition the protein can purified, or partially purified, from such culture or component (e.g., from culture medium or cell extracts or bodily fluid) using known processes. By "partially purified" means that some fractionation procedure, or procedures, have been carried out, but that more polypeptide species (at least 10%) than the desired protein is present. By "purified" is meant that the protein is essentially homogeneous, i.e., less than 1% contaminating proteins are present. Fractionation procedures can include but are not limited to one or more steps of filtration, centrifugation, precipitation, phase separation, affinity purification, gel filtration, ion exchange chromatography, hydrophobic interaction chromatography (HIC; using such resins as phenyl ether, butyl ether, or propyl ether), HPLC, or some combination of above.

For example, the purification of the polypeptide can include an affinity column containing agents which will bind to the polypeptide; one or more column steps over such affinity resins as concanavalin A-agarose, HEPARIN-TOYOPEARL (chromatography medium) or Cibacrom blue 3GA SEPHAROSE (agarose beads); one or more steps involving elution; and/or immunoaffinity chromatography. The polypeptide can be expressed in a form that facilitates purification. For example, it may be expressed as a fusion polypeptide, such as those of maltose binding polypeptide (MBP), glutathione-S-transferase (GST) or thioredoxin (TRX). Kits for expression and purification of such fusion polypeptides are commercially available from New England BioLab (Beverly, Mass.), Pharmacia (Piscataway, N.J.) and InVitrogen, respectively. The polypeptide can be tagged with an epitope and subsequently purified by using a specific antibody directed to such epitope. One such epitope FLAG (epitope tag) is commercially available from Kodak (New Haven, Conn.). It is also possible to utilize an affinity column comprising a polypeptide-binding polypeptide, such as a monoclonal antibody to the recombinant protein, to affinity-purify expressed polypeptides. Other types of affinity purification steps can be a Protein A or a Protein G column, which affinity agents bind to proteins that contain Fc domains. Polypeptides can be removed from an affinity column using conventional techniques, e.g., in a high salt elution buffer and then dialyzed into a lower salt buffer for use or by changing pH or other components depending on the affinity matrix utilized, or can be competitively removed using the naturally occurring substrate of the affinity moiety.

The desired degree of final purity depends on the intended use of the polypeptide. A relatively high degree of purity is desired when the polypeptide is to be administered in vivo, for example. In such a case, the polypeptides are purified such that no polypeptide bands corresponding to other polypeptides are detectable upon analysis by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). It will be recognized by one skilled in the pertinent field that multiple bands corresponding to the polypeptide can be visualized by SDS-PAGE, due to differential glycosylation, differential post-translational processing, and the like. Most preferably, the polypeptide of the invention is purified to substantial homogeneity, as indicated by a single polypeptide band upon analysis by SDS-PAGE. The polypeptide band can be visualized by silver staining, Coomassie blue staining, or (if the polypeptide is radiolabeled) by autoradiography.

The invention also optionally encompasses further formulating the proteins. By the term "formulating" is meant

that the proteins can be buffer exchanged, sterilized, bulk-packaged and/or packaged for a final user. For purposes of the invention, the term "sterile bulk form" means that a formulation is free, or essentially free, of microbial contamination (to such an extent as is acceptable for food and/or drug purposes), and is of defined composition and concentration. The term "sterile unit dose form" means a form that is appropriate for the customer and/or patient administration or consumption. Such compositions can comprise an effective amount of the protein, in combination with other components such as a physiologically acceptable diluent, carrier, or excipient. The term "physiologically acceptable" means a non-toxic material that does not interfere with the effectiveness of the biological activity of the active ingredient(s).

Formulations suitable for administration include aqueous and non-aqueous sterile injection solutions which may contain anti-oxidants, buffers, bacteriostats and solutes which render the formulation isotonic with the blood of the recipient; and aqueous and non-aqueous sterile suspensions which may include suspending agents or thickening agents. The polypeptides can be formulated according to known methods used to prepare pharmaceutically useful compositions. They can be combined in admixture, either as the sole active material or with other known active materials suitable for a given indication, with pharmaceutically acceptable diluents (e.g., saline, Tris-HCl, acetate, and phosphate buffered solutions), preservatives (e.g., thimerosal, benzyl alcohol, parabens), emulsifiers, solubilizers, adjuvants and/or carriers. Suitable formulations for pharmaceutical compositions include those described in *Remington's Pharmaceutical Sciences,* 16th ed. 1980, Mack Publishing Company, Easton, Pa. In addition, such compositions can be complexed with polyethylene glycol (PEG), metal ions, or incorporated into polymeric compounds such as polyacetic acid, polyglycolic acid, hydrogels, dextran, etc., or incorporated into liposomes, microemulsions, micelles, unilamellar or multilamellar vesicles, erythrocyte ghosts or spheroblasts. Suitable lipids for liposomal formulation include, without limitation, monoglycerides, diglycerides, sulfatides, lysolecithin, phospholipids, saponin, bile acids, and the like. Preparation of such liposomal formulations is within the level of skill in the art, as disclosed, for example, in U.S. Pat. No. 4,235,871; U.S. Pat. No. 4,501,728; U.S. Pat. No. 4,837,028; and U.S. Pat. No. 4,737,323. Such compositions will influence the physical state, solubility, stability, rate of in vivo release, and rate of in vivo clearance, and are thus chosen according to the intended application, so that the characteristics of the carrier will depend on the selected route of administration. Sustained-release forms suitable for use include, but are not limited to, polypeptides that are encapsulated in a slowly-dissolving biocompatible polymer (such as the alginate microparticles described in U.S. Pat. No. 6,036,978), admixed with such a polymer (including topically applied hydrogels), and or encased in a biocompatible semi-permeable implant.

The invention having been described, the following examples are offered by way of illustration, and not limitation.

## EXAMPLE

### Amino Acid Feeds

During batch fed culture for production of a recombinant TNFR:Fc protein, a concentrated solution of amino acids was added to CHO cells under production conditions. The amino acid feed is conveniently added in two different buffers—a high pH solution to solubilize most amino acids, and a low pH solution to solubilize cystine and tyrosine. It was noticed that performance of the cell culture seemed to be improved when the low pH solution was buffered with phosphoric acid instead of hydrochloric acid. Accordingly, additional experimentation was performed to investigate the effect of phosphate in the amino acid feeds.

## EXAMPLE

### Addition of Phosphate Improves CHO Cell Culture Performance

In this experiment, the effect of altering the following three conditions was examined during production of a recombinant TNFR:Fc protein from CHO cells. The conditions that were tested were the following:

1. No phosphate or 2.5 mM $KH_2PO_4$ in amino acid feeds on days 4, 6 and 8.
2. 100 ul IGF-1 or 25 uL IGF-1 with 20 uM vanadate in the media.
3. A single day 2 feed, or the day 2 feed split into two halves and fed on day 1 and day 2.

Materials and Methods

Eight 2 liter production tanks with a 1 liter working volume (Applikon, Foster City, Calif.) were setup to investigate the effect of each combination of the three variables. Cells were seeded at about 7×10E5 cells per mL of medium with the indicated concentrations of vanadate and IGF-1 (Long [R3] IGF-1; GroPep, Australia). Growth was arrested by addition of sodium butyrate to 0.25 mM and incubation at 31 degrees C. The day 2 feed was a 15 fold concentrated serum-free complete medium. The days 4, 6, and 8 amino acid feed was a solution of 17 essential amino acids added as a 56× low pH amino acid feed (containing amino acids solubilized in a low pH buffer) and a 560× high pH amino acid feed (containing amino acids cystine and tyrosine, at a pH of about 12). When phosphate was added with the feed, it was present in the low pH amino acid solution.

The culture was maintained for 10 days, and samples taken daily to assay percent viability, viable cell density, and recombinant protein titer.

Results

FIG. **1** is a graph of the viable cell density (VCD) over time, and FIG. **2** is a graph of the percent viability over time, for each of the 8 different tanks.

Although there was no significant difference in performance between the tanks that contained vanadate and those with no vanadate, addition of vanadate allowed a reduction in the amount of IGF-1 required. Reduction of IGF-1 in presence of vanadate is very desirable, as IGF-1 is a very expensive media component. No performance difference was observed between tanks with one day 2 feed and those with the split day 2 feed.

The effect of adding phosphate in the feeds was enormous. The cells in the tanks, which were fed with phosphate, grew to higher cell density than in the tanks without phosphate. Besides growing to higher cell density, viability also remained higher in the phosphate fed tanks. Furthermore, the resulting titers of TNFR:Fc protein were 65% higher in the phosphate fed tanks.

In conclusion, addition of phosphate in the feed sufficient to make the cell culture 2.5 mM phosphate immediately after addition caused a dramatic increase in recombinant protein production.

The present invention is not to be limited in scope by the specific embodiments described herein, which are intended as single illustrations of individual aspects of the invention, and functionally equivalent methods and components are within the scope of the invention. Indeed, various modifications of the invention, in addition to those shown and described herein will become apparent to those skilled in the art from the foregoing description and accompanying drawings. Such modifications are intended to fall within the scope of the appended claims.

What is claimed is:

**1**. A method of producing a recombinant protein, the method comprising culturing a Chinese Hamster Ovary (CHO) cell culture genetically engineered to produce the protein in a tissue culture medium, and adding a feed solution to the cell culture, wherein the feed solution comprises a phosphate compound, where the phosphate compound is added in an amount sufficient to achieve a final cell culture concentration of from 1.5 millimolar to 3.5 millimolar phosphate, and wherein production of the recombinant protein by the cell culture is increased as compared to the CHO cell culture in the absence of the phosphate compound in the feed solution.

**2**. The method of claim **1**, wherein the feed solution additionally comprises one or more amino acids.

**3**. The method of claim **1**, wherein the phosphate compound is selected from the group consisting of sodium phosphate, potassium phosphate, phosphoric acid, and other salts of phosphoric acid.

**4**. The method of claim **2**, wherein the phosphate compound is added as a component of an amino acid feed.

**5**. The method of claim **2**, wherein the feed solution is added repeatedly.

**6**. The method of claim **5**, wherein the feed solution is added about every two days.

**7**. The method of claim **1**, wherein the cells are under inducing conditions when the feed solution is added.

**8**. The method of claim **7** wherein the inducing conditions comprise at least one condition selected from the group consisting of a reduction in temperature, an addition of a sodium butyrate solution, an addition of dimethylsulfoxide (DMSO), and an addition of dimethylformamide (DMF).

**9**. The method of claim **1**, wherein the protein is a soluble form of a human tumor necrosis factor (TNF) receptor.

**10**. The method of claim **1**, wherein size of the culture is at least about 100 liters.

**11**. The method of claim **10**, wherein the size of the culture is at least about 1000 liters.

**12**. The method of claim **1**, wherein the tissue culture medium is serum-free.

**13**. The method of claim **1**, further comprising collecting the protein.

**14**. The method of claim **13**, further comprising formulating the protein.

**15**. A method of producing a recombinant protein, the method comprising culturing a Chinese Hamster Ovary (CHO) cell culture genetically engineered to produce a protein in a tissue culture medium under induction conditions, and adding a feed solution to the cell culture, wherein the feed solution comprises an amount of a phosphate compound sufficient to bring the medium to about 2.5 mM phosphate after addition, and wherein production of the recombinant protein by the cell culture is increased as compared to the CHO cell culture in the absence of the phosphate compound in the feed solution.

**16**. The method of claim **15**, further comprising adding one or more amino acids to the cell culture.

**17**. The method of claim **16**, further comprising collecting the protein.

**18**. The method of claim **17**, further comprising at least partially purifying the protein.

* * * * *

# EXHIBIT 5

US007157557B2

(12) **United States Patent**
**Sassenfeld et al.**

(10) **Patent No.: US 7,157,557 B2**
(45) **Date of Patent: Jan. 2, 2007**

(54) **INCREASED RECOVERY OF ACTIVE PROTEINS**

(75) Inventors: **Helmut M. Sassenfeld**, Bainbridge Island, WA (US); **Richard L. Remmele, Jr.**, Lynnwood, WA (US); **Rebecca E. McCoy**, Seattle, WA (US)

(73) Assignee: **Immunex Corporation**, Thousand Oaks, CA (US)

( * ) Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 221 days.

(21) Appl. No.: **10/080,471**

(22) Filed: **Feb. 22, 2002**

(65) **Prior Publication Data**

US 2002/0182665 A1 Dec. 5, 2002

**Related U.S. Application Data**

(60) Provisional application No. 60/271,033, filed on Feb. 23, 2001.

(51) **Int. Cl.**
***C07K 14/715*** (2006.01)

(52) **U.S. Cl.** ..... **530/350**; 530/412; 530/413; 530/417; 530/247; 514/12; 435/325; 435/69.1; 435/69.7; 536/23.1; 536/23.4; 536/23.5

(58) **Field of Classification Search** ..... 530/350, 530/417; 514/12; 435/69.1
See application file for complete search history.

(56) **References Cited**

U.S. PATENT DOCUMENTS

| | | | | |
|---|---|---|---|---|
| 4,766,205 | A | 8/1988 | Ghosh-Dastidar | |
| 5,447,851 | A * | 9/1995 | Beutler et al. | 435/69.7 |
| 5,661,001 | A * | 8/1997 | Grossenbacher et al. | 435/69.1 |
| 5,879,673 | A * | 3/1999 | Thomas | 424/85.1 |
| 2003/0099934 | A1 | 5/2003 | Boudet et al. | |

FOREIGN PATENT DOCUMENTS

| | | | |
|---|---|---|---|
| EP | 0 293 785 | A2 | 5/1988 |
| EP | 0 293 785 | * | 12/1988 |
| EP | 0 553 494 | A1 | 12/1992 |
| EP | 0433225 | B1 | 4/1999 |
| WO | WO 95/32216 | | 11/1995 |
| WO | WO 96/03141 | A1 | 2/1996 |
| WO | WO 01/34638 | A1 | 5/2001 |
| WO | WO 01/49720 | A2 | 7/2001 |

OTHER PUBLICATIONS

Merli et al., Analytical Biochemistry, Sep. 1, 1995, vol. 320, No. 1, pp. 85-91.*
The Cytokine Facts Book, Second Edition, Academic Press, 2001, pp. 476-478.*
Flamand et al., "Purification and Renaturation of Japanese Encephalitis Virus Nonstructural Glycoprotein NS1 Overproduced by Insect Cells," *Protein Expression and Purification* 6:519-527, 1995.
Creighton, "Disulphide Bonds and Protein Stability," *BioEssays* 8(2):57-63, 1988.
International Search Report, PCT/US02/05645, mailed Jun. 17, 2003.
U.S. Appl. No. 11/255,528, Oct. 21, 2005, Dillon et al.

* cited by examiner

*Primary Examiner*—Eileen B. O'Hara
(74) *Attorney, Agent, or Firm*—Kathleen Fowler

(57) **ABSTRACT**

The invention provides methods of increasing yields of desired conformation of proteins. In particular embodiments, the invention includes contacting preparations of a recombinant protein with a reduction/oxidation coupling reagent for a time sufficient to increase the relative proportion of a desired configurational isomer.

**49 Claims, 7 Drawing Sheets**

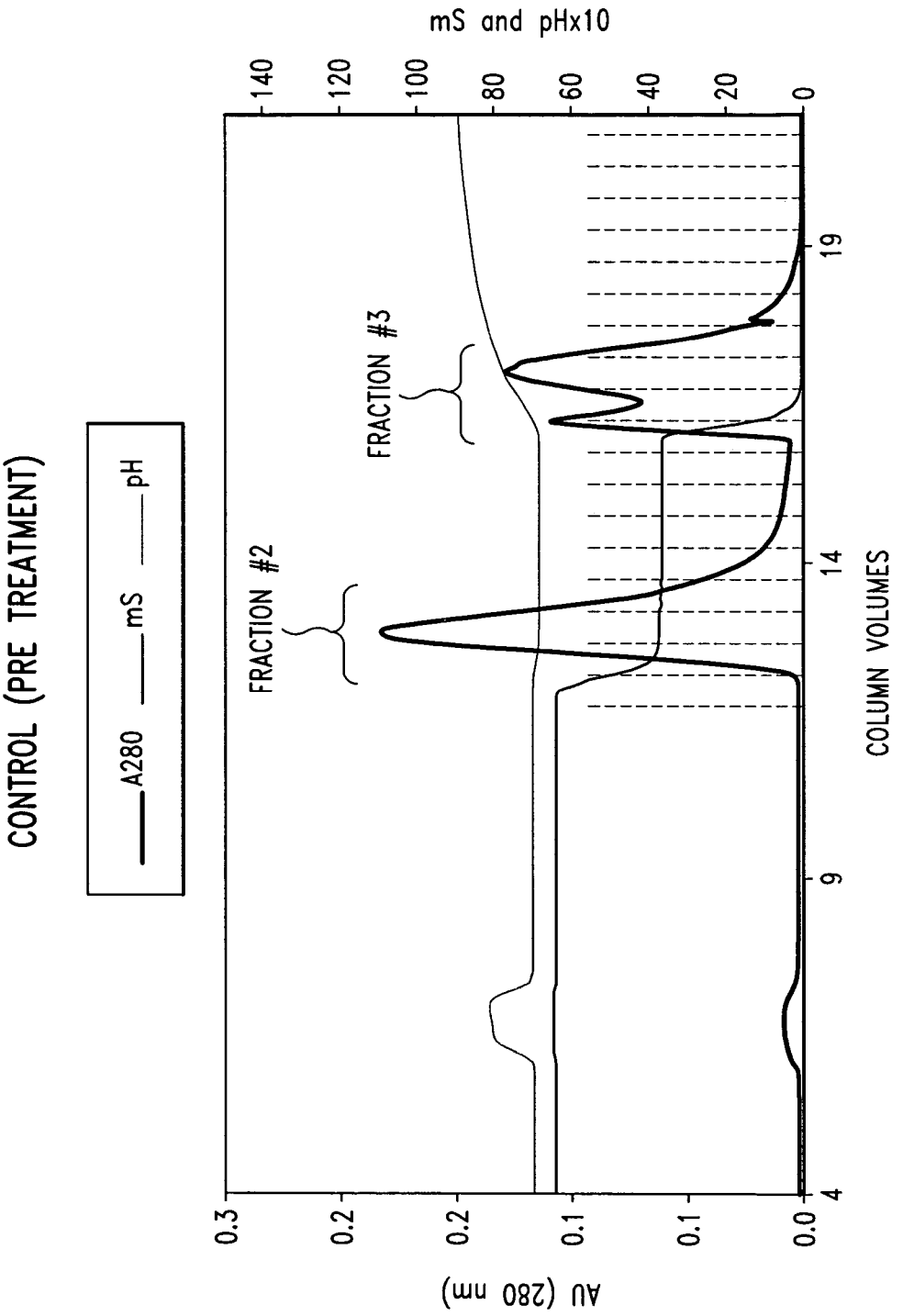

CONTROL (PRE TREATMENT)
A280
mS
pH
mS and pHx10
140
120
100
80
60
40
20
0
FRACTION #2
FRACTION #3
19
14
9
4
COLUMN VOLUMES
0.3
0.2
0.2
0.1
0.1
0.0
AU (280 nm)

*Fig. 1*

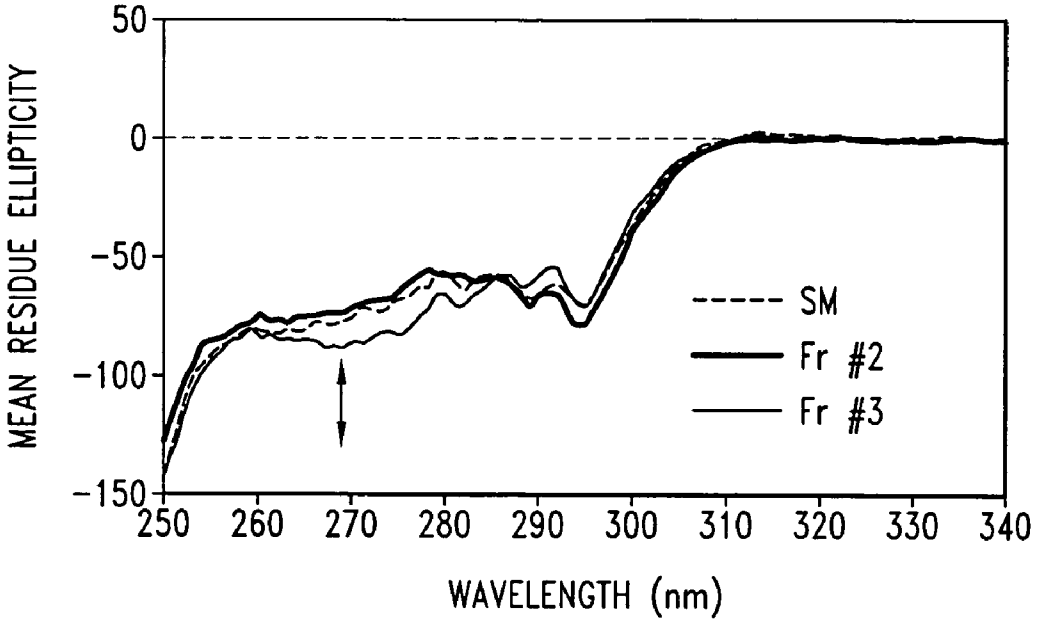

MEAN RESIDUE ELLIPTICITY
50
0
-50
-100
-150
250
260
270
280
290
300
310
320
330
340
WAVELENGTH (nm)
SM
Fr #2
Fr #3

*Fig. 2A*

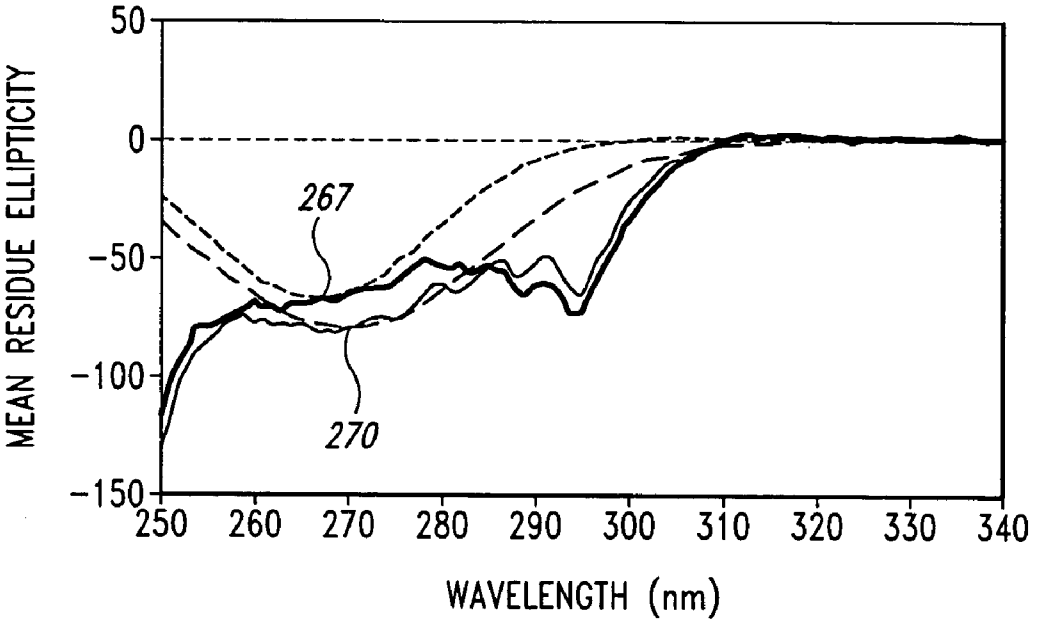

MEAN RESIDUE ELLIPTICITY
50
0
-50
-100
-150
267
270
250
260
270
280
290
300
310
320
330
340
WAVELENGTH (nm)

*Fig. 2B*

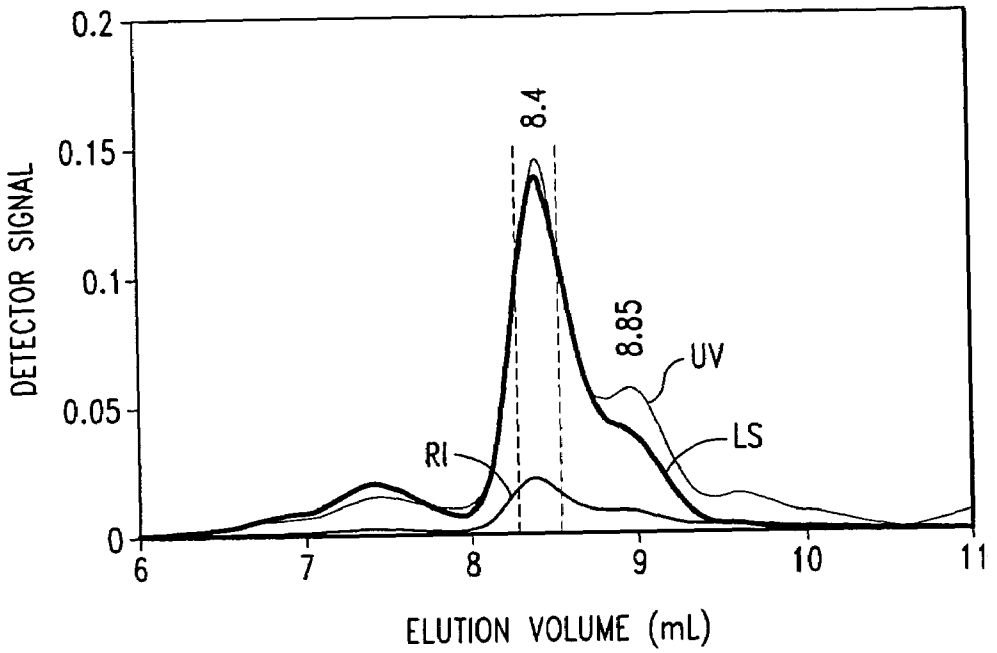

0.2
0.15
0.1
0.05
0
DETECTOR SIGNAL
8.4
8.85
UV
LS
RI
6
7
8
9
10
11
ELUTION VOLUME (mL)

*Fig. 3A*

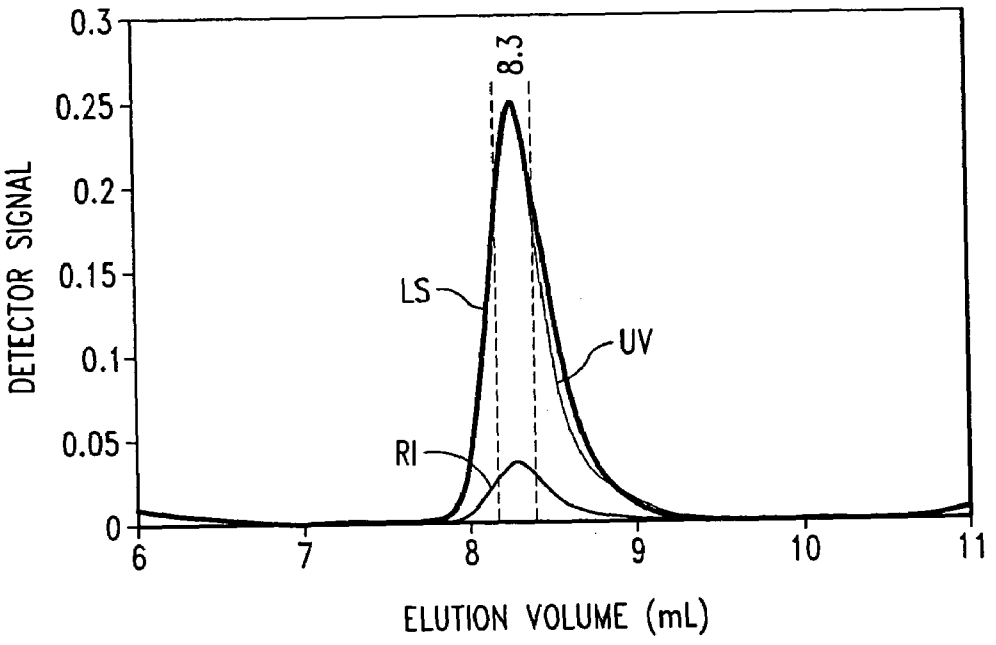

0.3
0.25
0.2
0.15
0.1
0.05
0
DETECTOR SIGNAL
8.3
LS
UV
RI
6
7
8
9
10
11
ELUTION VOLUME (mL)

*Fig. 3B*

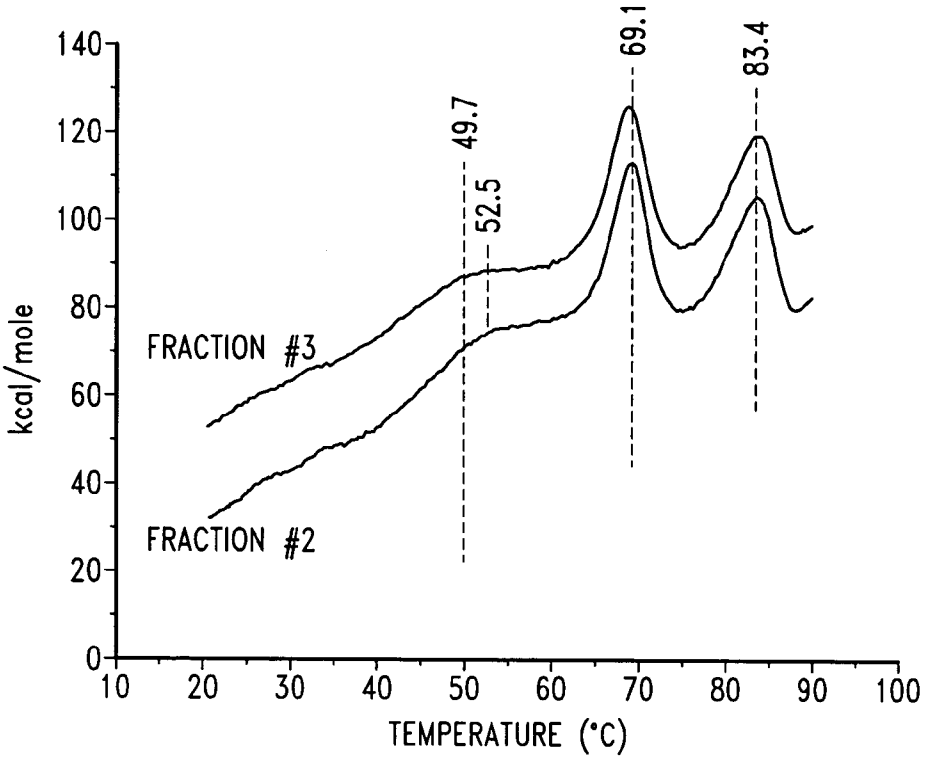

140
120
100
80
60
40
20
0
kcal/mole
10
20
30
40
50
60
70
80
90
100
TEMPERATURE (°C)
49.7
52.5
69.1
83.4
FRACTION #3
FRACTION #2

*Fig. 4A*

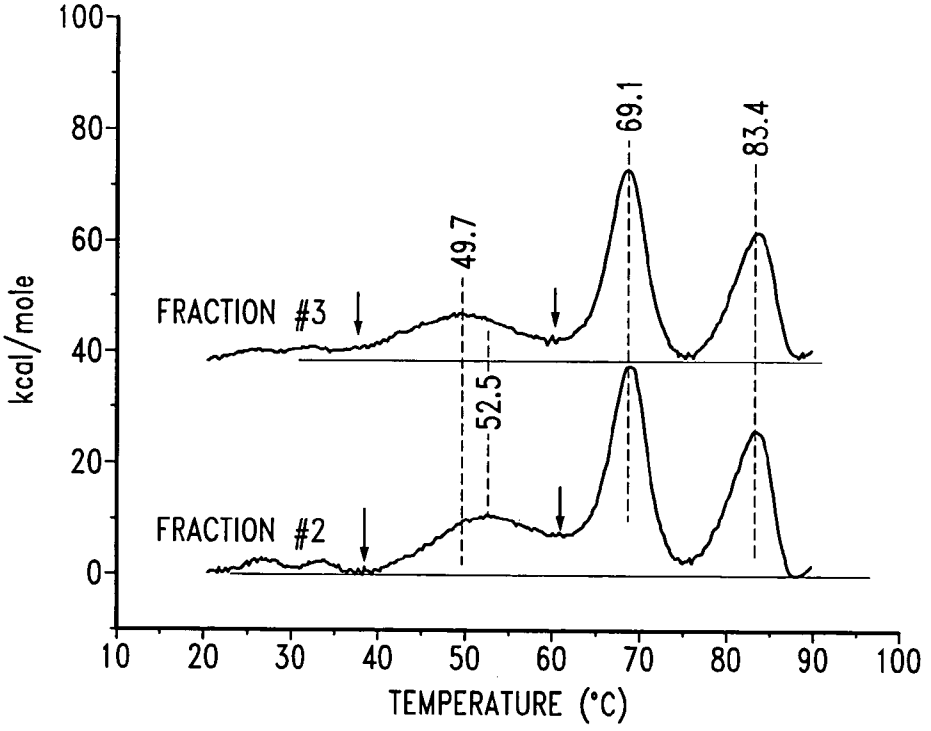

100
80
60
40
20
0
kcal/mole
10
20
30
40
50
60
70
80
90
100
TEMPERATURE (°C)
49.7
52.5
69.1
83.4
FRACTION #3
FRACTION #2

*Fig. 4B*

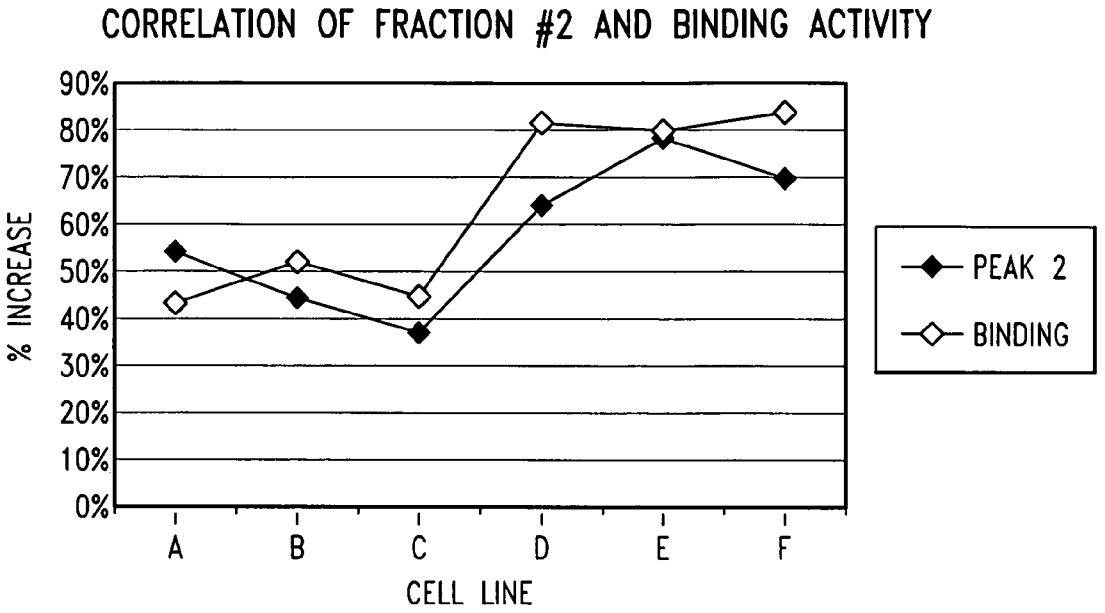

CORRELATION OF FRACTION #2 AND BINDING ACTIVITY
% INCREASE
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
A
B
C
D
E
F
CELL LINE
PEAK 2
BINDING

*Fig. 5*

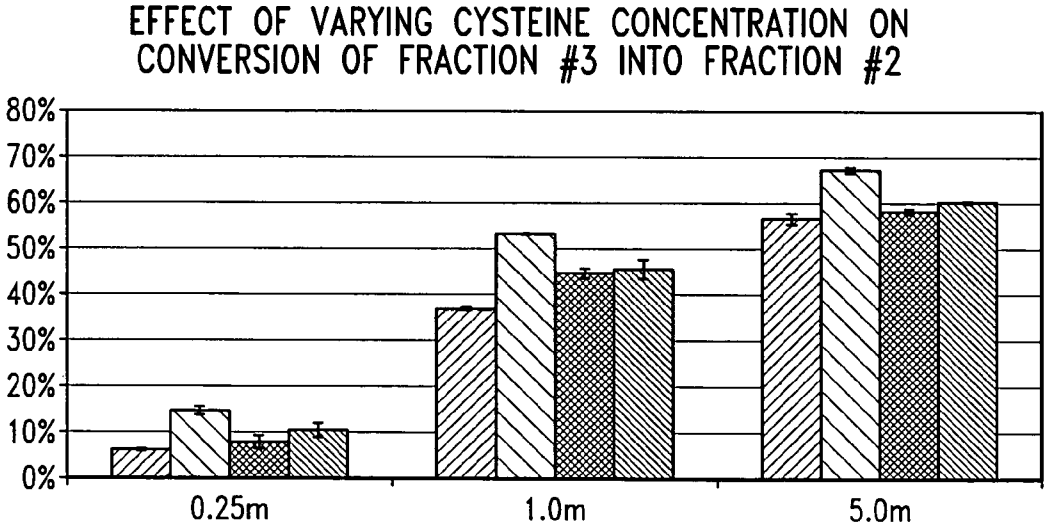

EFFECT OF VARYING CYSTEINE CONCENTRATION ON CONVERSION OF FRACTION #3 INTO FRACTION #2
80%
70%
60%
50%
40%
30%
20%
10%
0%
0.25m
1.0m
5.0m

*Fig. 6*

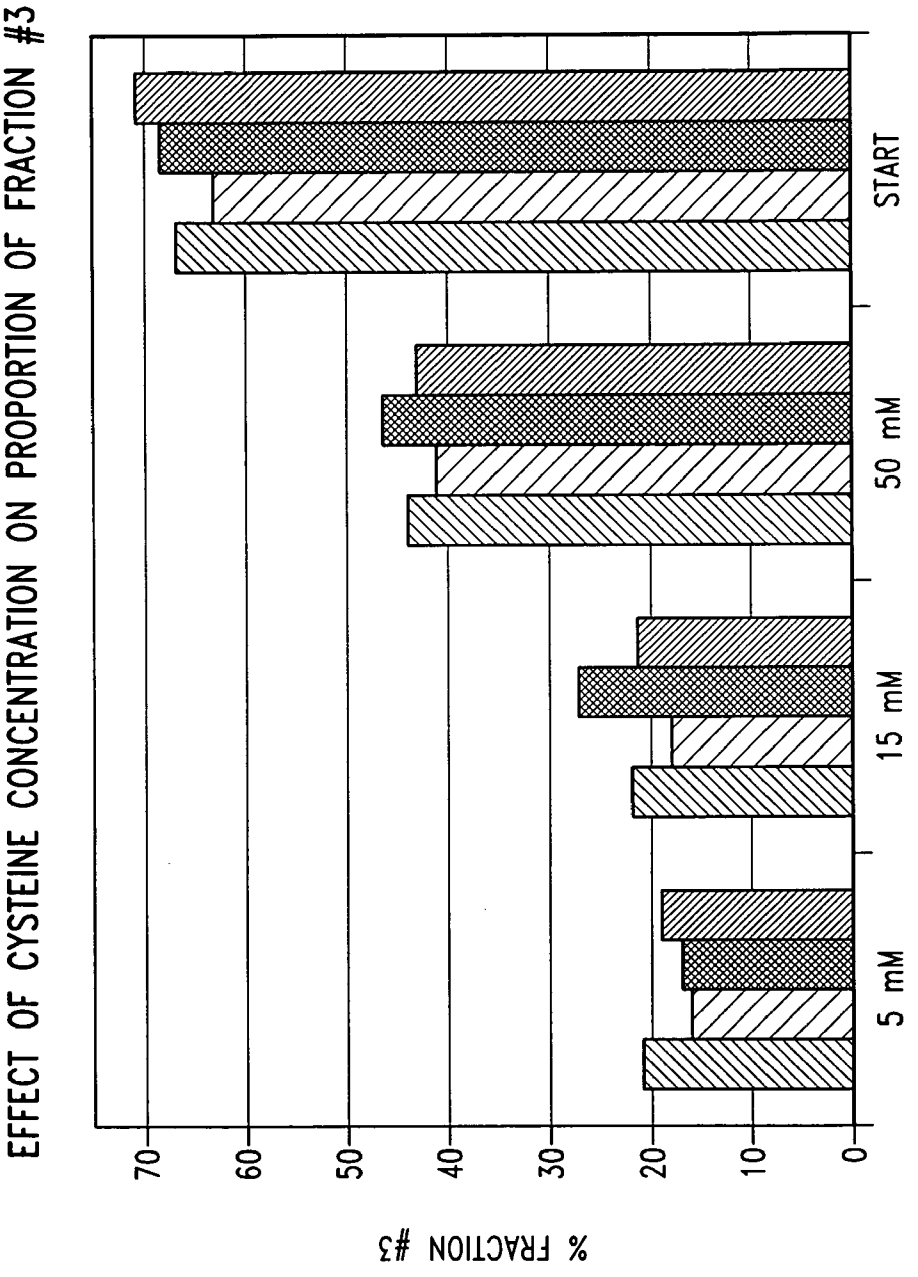

EFFECT OF CYSTEINE CONCENTRATION ON PROPORTION OF FRACTION #3
% FRACTION #3
0
10
20
30
40
50
60
70
START
50 mM
15 mM
5 mM

*Fig. 7*

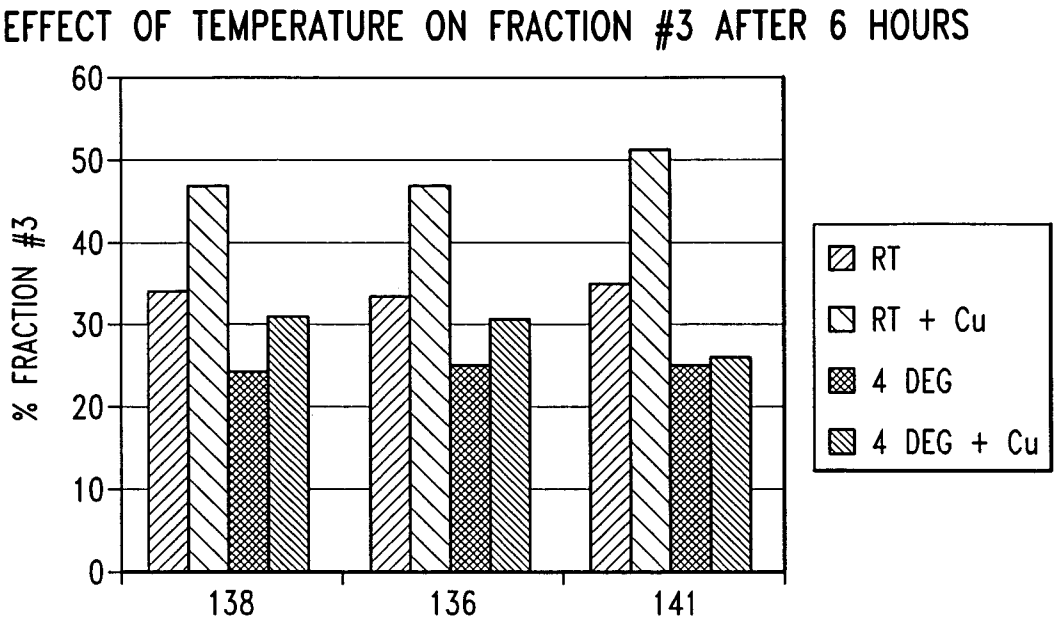

EFFECT OF TEMPERATURE ON FRACTION #3 AFTER 6 HOURS
% FRACTION #3
60
50
40
30
20
10
0
138
136
141
RT
RT + Cu
4 DEG
4 DEG + Cu

*Fig. 8A*

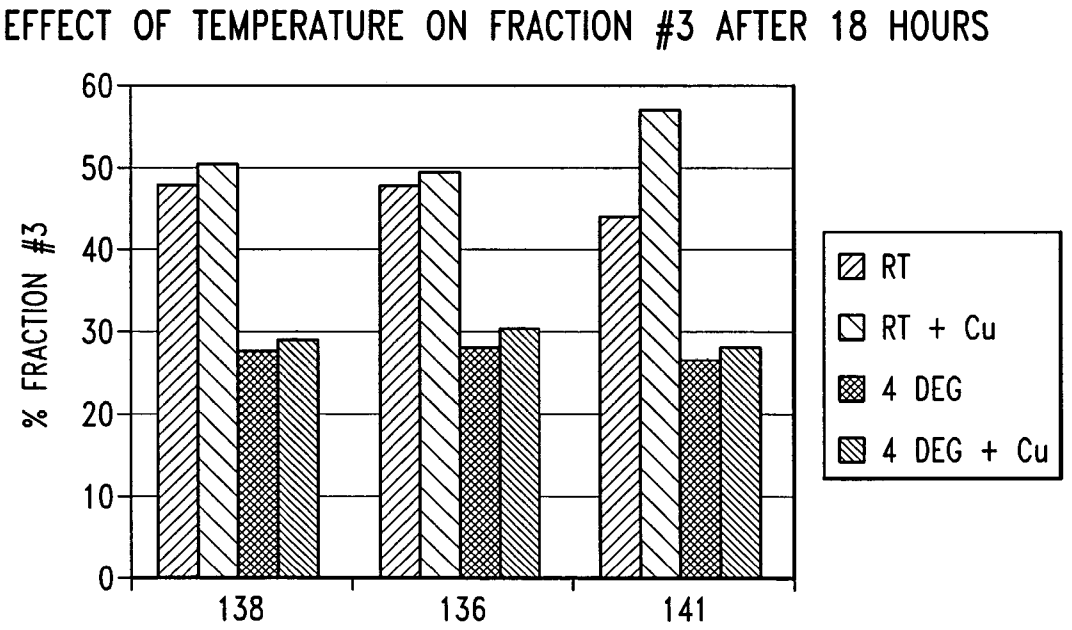

EFFECT OF TEMPERATURE ON FRACTION #3 AFTER 18 HOURS
% FRACTION #3
60
50
40
30
20
10
0
138
136
141
RT
RT + Cu
4 DEG
4 DEG + Cu

*Fig. 8B*

# INCREASED RECOVERY OF ACTIVE PROTEINS

This application claims the benefit of provisional U.S. application 60/271,033, filed Feb. 23, 2001, the disclosure of which is incorporated by reference in its entirety.

## FIELD OF THE INVENTION

The invention is in the field of treatment and purification of proteins.

## BACKGROUND

High levels of expression of many proteins of eukaryotic origin have been achieved in prokaryotic expression hosts. Such eukaryotic proteins often misfold and accumulate as insoluble inclusion bodies in the prokaryotic host. In order to obtain biologically active protein, the proteins trapped in inclusion bodies had to be unfolded and refolded under harsh conditions including chaotropic agents and reducing thiols.

Expression of proteins of eukaryotic origin in eukaryotic hosts avoided these problems. Provided that the expression vector was properly designed (e.g., with secretory signal peptides, etc.), eukaryotic cell lines tend to correctly process and secrete extracellular eukaryotic proteins as soluble products.

However, as expression systems and vectors have been improved to maximize levels of expression from eukaryotic hosts, not all of the recombinant protein expressed and secreted from these hosts is in the desired, most active conformation. The invention is designed to overcome such expression problems, and maximize yields of biologically active protein.

## SUMMARY OF THE INVENTION

The invention is based, in part, on the discovery that not all of the preparation of recombinant protein that is expressed by eukaryotic host cells is folded into a native tertiary conformation. In addition, it has been found that regions or domains of recombinant proteins may be properly folded, while other regions or domains may have undesired conformations. Accordingly, in one aspect, the invention provides a method of contacting a preparation of the recombinant protein that contains a mixture of at least two isomers of the recombinant protein to a reduction/oxidation coupling reagent for a time sufficient to increase the relative proportion of the desired conformational isomer and determining the relative proportion of the desired conformational isomer in the mixture. In another aspect, the invention entails contacting a preparation of a recombinant protein that has been produced by mammalian cells with a reduction/oxidation coupling reagent, at a pH of about 7 to about 11, and isolating a fraction of the preparation of the recombinant protein with a desired conformation. Preferred recombinant proteins are glycosylated recombinant proteins such as, e.g., those produced by eukaryotic cells. The invention also relates to methods of formulating the resulting preparations into a sterile unit dose form, and compositions produced by the methods of the invention.

## BRIEF DESCRIPTION OF THE FIGURES

FIG. **1**. Hydrophobic interaction chromatography (HIC) of TNFR:Fc. This preparation of TNFR:Fc elutes during HIC as three distinct peaks collected into Fraction #2 and Fraction #3, as indicated.



FIG. **2**. Circular Dichroism Analysis of Fractions #2 and #3. Near-UV Circular Dichroism measurements expressed in terms of mean residue ellipticity are shown in FIG. **2**. FIG. **2**A presents the spectral data; The line for Fraction #3 is closest to the arrow highlighting the negative displacement at about 270 nM ascribed to disulfide contributions, and the line for Fraction #2 is the darker solid line. FIG. **2**B presents the curve-fitted data for Fraction #2 (small dashed line) and Fraction #3 (larger dashed line).

FIG. **3**. Molecular Weight Determination Using On-line size exclusion chromatography (SEC), ultraviolet (UV), light scattering (LS), and refractive index (RI) detection in series (On-line SEC/UV/LS/RI). FIG. **3**A is Fraction #3, and FIG. **3**B is Fraction #2. Vertical dashed lines indicate where the slices were evaluated for molecular weight determination in the region surrounding the main peak.

FIG. **4**. Differential Scanning Calorimetry Analysis of Fractions #2 and #3. FIG. **4**A is the uncorrected data, and FIG. **4**B presents the baseline-corrected data. Thermal melting transitions are labeled by vertical dashed lines. Arrows indicate an enthalpy displacement. The horizontal dotted lines in FIG. **4**B are used as a baseline reference.

FIG. **5**. Correlation of Fraction #2 and Binding Activity. Six different preparations of TNFR:Fc (denoted A through F), from six different cell lines, were tested for the correlation between the percent increase in proportion of Fraction #2 (dark diamonds) and percent increase in TNF alpha Binding Units (light diamonds).

FIG. **6**. Effect of Varying Cysteine Concentration on Conversion of Fraction #3 into Fraction #2. Protein samples were treated with various concentrations of cysteine (0.25–5.0 mM) and changes in Fraction #3 assessed using HIC. Four different lots of TNFR:Fc were treated for 18 hours at the indicated cysteine concentration on the x-axis. The percent of Fraction #3 in each lot that was converted into Fraction #2 is plotted on the y-axis.

FIG. **7**. Effect of Cysteine Concentration on Proportion of Fraction #3. Protein samples from four different lots were treated with various concentrations of cysteine (0–50 mM) and the resulting level of Fraction #3 was assessed by HIC.

FIG. **8**. Effect of Temperature on Disulfide Exchange. Protein fractions were treated at room temperature or 4 degrees C. in the presence or absence of copper for various times. FIG. **8**A presents changes in HIC Fraction #3 after 6 Hours, and FIG. **8**B presents changes in HIC Fraction #3 after 18 hours.

## DETAILED DESCRIPTION OF THE INVENTION

The invention provides methods of increasing the recovery of active recombinant proteins. In particular, the invention involves promoting a desired conformation of a protein in preparations of a recombinant protein. Significantly, the invention provides gentle methods of altering protein structure without necessitating the use of harsh chaotrope treatments (such as, for example, strong denaturants such as SDS, guanidium hydrochloride or urea). Using the methods of the invention on preparations of recombinant protein results in a higher percentage, or higher relative fraction, of the recombinant protein in the preparation with a desired conformation. A desired conformation for a recombinant protein is the three-dimensional structure of a protein that most closely resembles, and/or duplicates the function of, the naturally occurring domain of that protein. Such gentle

methods are particularly advantageous when the recombinant protein is intended to be used in vivo as a drug or biologic.

Generally, when the recombinant protein contains a domain of a receptor protein, the desired conformation will have a higher binding affinity (and, consequently, a lower dissociation constant) for a cognate ligand of the receptor. For example, the desired conformation of a TNF-binding molecule will have a higher binding affinity and a lower dissociation constant for TNF (e.g., TNF-alpha).

In addition, the desired conformation of a recombinant protein is preferably more thermostable than an undesired conformation (when measured in the same solution environment). Thermostability can be measured in any of a number of ways such as, for example, the melting temperature transition (Tm). The desired conformation of a recombinant protein may or may not have a different arrangement of disulfide bonds, although preferably the conformation contains native disulfide bonds. The desired conformation of a recombinant protein may have other tertiary structure characteristics. For example, a desired conformation may be a monomer, dimer, trimer, tetramer, or some other higher order form of the protein. For the purposes of the invention, the "conformation" of a protein is its three-dimensional structure. Two different structures of a polypeptide with the same primary amino acid sequence are "conformers" of each other when they have different conformations corresponding to energy minima, and they differ from each other only in the way their atoms are oriented in space. Conformers can be interconverting (referring to the rotational freedom around bonds to the exclusion of breaking bonds). Two different structures of a polypeptide with the same primary amino acid sequence are "configurational isomers" when they have different conformations corresponding to energy minima, they differ from each other in the way their atoms are oriented in space, and they are non-interconvertible without the breaking of a covalent bond. In the practice of the invention, configurational isomers can be interconverted by, for example, breaking and optionally reforming disulfidc bonds.

Thus, in one aspect, the invention entails contacting a preparation of the glycosylatcd recombinant protein that contains a mixture of at least two configurational isomers of the recombinant protein to a reduction/oxidation coupling reagent for a time sufficient to increase the relative proportion of the desired configurational isomer and determining the relative proportion of the desired configurational isomer in the mixture. In another aspect, the invention entails contacting a preparation of a recombinant protein that has been produced by mammalian cells with a reduction/oxidation coupling reagent, at a pH of about 7 to about 11, and isolating a fraction of the preparation of the recombinant protein with a desired conformation. Preferred recombinant proteins are glycosylated recombinant proteins such as, e.g., those produced by eukaryotic cells.

The invention can be used to treat just about any protein to promote a desired conformation. A protein is generally understood to be a polypeptide of at least about 10 amino acids, more preferably at least about 25 amino acids, even more preferably at least about 75 amino acids, and most preferably at least about 100 amino acids. The methods of the invention find particular use in treating proteins that have at least about 3 cysteine residues, more preferably at least about 8 cysteine residues, still more preferably at least about 15 cysteine residues, yet even more preferably at least about 30, still even more preferably at least about 50 to 150 cysteine residues.

Generally, the methods of the invention are useful for improving production processes for recombinant proteins. Recombinant proteins are proteins produced by the process of genetic engineering. The term "genetic engineering" refers to any recombinant DNA or RNA method used to create a host cell that expresses a gene at elevated levels, at lowered levels, and/or a mutant form of the gene. In other words, the cell has been transfected, transformed or transduced with a recombinant polynucleotide molecule, and thereby altered so as to cause the cell to alter expression of a desired protein. Methods and vectors for genetically engineering cells and/or cell lines to express a protein of interest are well known to those skilled in the art; for example, various techniques are illustrated in *Current Protocols in Molecular Biology*, Ausubel et al., eds. (Wiley & Sons, New York, 1988, and quarterly updates) and Sambrook et al., *Molecular Cloning: A Laboratory Manual* (Cold Spring Laboratory Press, 1989). Genetic engineering techniques include but are not limited to expression vectors, targeted homologous recombination and gene activation (see, for example, U.S. Pat. No. 5,272,071 to Chappel) and trans activation by engineered transcription factors (see, for example, Segal el al., 1999, Proc. Natl. Acad. Sci. USA 96(6):2758–63).

The invention finds particular use in improving the production of proteins that are glycosylated. Specifically, proteins that are secreted by fungal cell systems (e.g., yeast, filamentous fungi) and mammalian cell systems will be glycosylated. Preferably, the proteins are secreted by mammalian production cells adapted to grow in cell culture. Examples of such cells commonly used in the industry are CHO, VERO, BHK, HeLa, CV1 (including Cos), MDCK, 293, 3T3, myeloma cell lines (especially murine), PC12 and WI38 cells. Particularly preferred host cells are Chinese hamster ovary (CHO) cells, which are widely used for the production of several complex recombinant proteins, e.g. cytokines, clotting factors, and antibodies (Brasel et al., 1996, Blood 88:2004–2012; Kaufman et al., 1988, J.Biol Chem 263: 6352–6362; McKinnon et al., 1991, J Mol Endocrinol 6:231–239; Wood et al., 1990, J. Immunol 145:3011–3016). The dihydrofolate reductase (DHFR)-deficient mutant cell line (Urlaub et al., 1980, Proc Natl Acad Sci USA 77:4216–4220), DXB11 and DG-44, are the CHO host cell lines of choice because the efficient DHFR selectable and amplifiable gene expression system allows high level recombinant protein expression in these cells (Kaufman R. J., 1990, Meth Enzymol 185:527–566). In addition, these cells are easy to manipulate as adherent or suspension cultures and exhibit relatively good genetic stability. CHO cells and recombinant proteins expressed in them have been extensively characterized and have been approved for use in clinical manufacturing by regulatory agencies.

It has been found that the invention is a gentle and effective process for improving the production process for proteins that can adopt multiple conformations and/or contain more than one domain. A "domain" is a contiguous region of the polypeptide chain that adopts a particular tertiary structure and/or has a particular activity that can be localized in that region of the polypeptide chain. For example, one domain of a protein can have binding affinity for one ligand, and one domain of a protein can have binding affinity for another ligand. In a thermostable sense, a domain can refer to a cooperative unfolding unit of a protein. Such proteins that contain more than one domain can be found naturally occurring as one protein or genetically engineered as a fusion protein. In addition, domains of a polypeptide can have subdomains.

In one aspect, the methods of the invention can be used on preparations of recombinant proteins in which at least one domain of the protein has a stable conformation, and at least one domain of the protein has an unstable conformation. The terms "stable" and "unstable" are used as relative terms. The domain of the protein with a stable conformation will have, for example, a higher melting temperature (Tm) than the unstable domain of the protein when measured in the same solution. A domain is stable compared to another domain when the difference in the Tm is at least about 2° C., more preferably about 4° C., still more preferably about 7° C., yet more preferably about 10° C., even more preferably about 15° C., still more preferably about 20° C., even still more preferably about 25° C., and most preferably about 30° C., when measured in the same solution.

The invention is also generally applicable to proteins that have an Fc domain, and another domain (e.g., antibodies, and Fc fusion proteins). For example, in one of the non-limiting embodiments illustrated below, TNFR:Fc, the Tm's for the Fc portion of the molecule are at 69.1° C. and 83.4° C., while the Tm for the TNFR portion of the molecule range from 52.5° C. (in the more desired conformation) to a Tm of 49.7° C. (in the less desired conformation).

Particularly preferred proteins are protein-based drugs, also known as biologics. Preferably, the proteins are expressed as extracellular products. Proteins that can be produced using the methods of the invention include but are not limited to a flt3 ligand (as described in WO 94/28391, which is incorporated by reference herein in its entirety), a CD40 ligand (as described in U.S. Pat. No. 6,087,329, which is incorporated by reference herein in its entirety), erythropoeitin, thrombopoeitin, calcitonin, Fas ligand, ligand for receptor activator of NF-kappa B (RANKL), tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL, as described in WO 97/01633, which is incorporated by reference herein in its entirety), thymic stroma-derived lymphopoietin, granulocyte colony stimulating factor, granulocyte-macrophage colony stimulating factor (GM-CSF, as described in Australian Patent No. 588819, which is incorporated by reference herein in its entirety), mast cell growth factor, stem cell growth factor, epidermal growth factor, RANTES, growth hormone, insulin, insulinotropin, insulin-like growth factors, parathyroid hormone, interferons, nerve growth factors, glucagon, interleukins 1 through 18, colony stimulating factors, lymphotoxin-β, tumor necrosis factor (TNF), leukemia inhibitory factor, oncostatin-M, and various ligands for cell surface molecules ELK and Hek (such as the ligands for eph-related kinases or LERKS). Descriptions of proteins that can be purified according to the inventive methods may be found in, for example, *Human Cytokines: Handbook for Basic and Clinical Research, Vol. II* (Aggarwal and Gutterman, eds. Blackwell Sciences, Cambridge, Mass., 1998); *Growth Factors: A Practical Approach* (McKay and Leigh, eds., Oxford University Press Inc., New York, 1993); and *The Cytokine Handbook* (A. W. Thompson, ed., Academic Press, San Diego, Calif., 1991).

Preparations of the receptors, especially soluble forms of the receptors, for any of the aforementioned proteins can also be improved using the inventive methods, including both forms of TNFR (referred to as p55 and p75), Interleukin-1 receptors types I and II (as described in EP 0 460 846, U.S. Pat. No. 4,968,607, and U.S. Pat. No. 5,767,064, which are incorporated by reference herein in their entirety), Interleukin-2 receptor, Interleukin-4 receptor (as described in EP 0 367 566 and U.S. Pat. No. 5,856,296, which are incorporated by reference herein in their entirety), Interleukin-15 receptor, lnterleukin-17 receptor, Interleukin-18 receptor, granulocyte-macrophage colony stimulating factor receptor, granulocyte colony stimulating factor receptor, receptors for oncostatin-M and leukemia inhibitory factor, receptor activator of NF-kappa B (RANK, as described in U.S. Pat. No. 6,271,349, which is incorporated by reference herein in its entirety), receptors for TRAIL (including TRAIL receptors 1, 2, 3, and 4), and receptors that comprise death domains, such as Fas or Apoptosis-Inducing Receptor (AIR).

Other proteins whose production processes can be improved using the inventive methods include cluster of differentiation antigens (referred to as CD proteins), for example, those disclosed in *Leukocyte Typing VI* (*Proceedings of the VIth International Workshop and Conference*; Kishimoto, Kikutani et al., eds.; Kobe, Japan, 1996), or CD molecules disclosed in subsequent workshops. Examples of such molecules include CD27, CD30, CD39, CD40; and ligands thereto (CD27 ligand, CD30 ligand and CD40 ligand). Several of these are members of the TNF receptor family, which also includes 41BB and OX40; the ligands are often members of the TNF family (as are 41BB ligand and OX40 ligand); accordingly, members of the TNF and TNFR families can also be produced using the present invention.

Proteins that are enzymatically active can also be prepared according to the instant invention. Examples include metalloproteinase-disintegrin family members, various kinases, glucocerebrosidase, superoxide dismutase, tissue plasminogen activator, Factor VIII, Factor IX, apolipoprotein E, apolipoprotein A-I, globins, an IL-2 antagonist, alpha-1 antitrypsin, TNF-alpha Converting Enzyme, and numerous other enzymes. Ligands for enzymatically active proteins can also be expressed by applying the instant invention.

The inventive compositions and methods are also useful for preparation of other types of recombinant proteins, including immunoglobulin molecules or portions thereof, and chimeric antibodies (e.g., an antibody having a human constant region coupled to a murine antigen binding region) or fragments thereof. Numerous techniques are known by which DNA encoding immunoglobulin molecules can be manipulated to yield DNAs capable of encoding recombinant proteins such as single chain antibodies, antibodies with enhanced affinity, or other antibody-based polypeptides (see, for example, Larrick et al., 1989, Biotechnology 7:934–938; Reichmann et al., 1988, Nature 332:323–327; Roberts et al., 1987, Nature 328:731–734; Verhoeyen et al., 1988, Science 239:1534–1536; Chaudhary et al., 1989, Nature 339:394–397). Preparations of fully human antibodies (such as are prepared using transgenic animals, and optionally further modified in vitro), as well as humanized antibodies, can also be used in the invention. The term humanized antibody also encompasses single chain antibodies. See, e.g., Cabilly el al., U.S. Pat. No. 4,816,567; Cabilly et al., European Patent No. 0,125,023 B1; Boss et al., U.S. Pat. No. 4,816,397; Boss et al., European Patent No. 0,120, 694 B1; Neuberger, M. S. et al., WO 86/01533; Neuberger, M. S. etal., European Patent No. 0,194,276 B1; Winter, U.S. Pat. No. 5,225,539; Winter, European Patent No. 0,239,400 B1; Queen et al., European Patent No. 0 451 216 B1; and Padlan, E. A. et al., EP 0 519 596 A1. The method of the invention may also be used during the preparation of conjugates comprising an antibody and a cytotoxic or luminescent substance. Such substances include: maytansine derivatives (such as DM1); enterotoxins (such as a Staphlyococcal enterotoxin); iodine isotopes (such as iodine-125); technium isotopes (such as Tc-99m); cyanine fluorochromes (such as Cy5.5.18); and ribosome-inactivating proteins (such as bouganin, gelonin, or saporin-S6).

Examples of antibodies or antibody/cytotoxin or antibody/luminophore conjugates contemplated by the invention include those that recognize any one or combination of the above-described proteins and/or the following antigens: CD2, CD3, CD4, CD8, CD11a, CD14, CD18, CD20, CD22, CD23, CD25, CD33, CD40, CD44, CD52, CD80 (B7.1), CD86 (B7.2), CD147, IL-1α, IL-1β, IL-4, IL-5, IL-8, IL-10, IL-2 receptor, IL-4 receptor, IL-6 receptor, IL-13 receptor, IL-18 receptor subunits, PDGF-β, VEGF, TGF, TGF-β2, TGF-β1, EGF receptor, VEGF receptor, C5 complement, IgE, tumor antigen CA125, tumor antigen MUC1, PEM antigen, LCG (which is a gene product that is expressed in association with lung cancer), HER-2, a tumor-associated glycoprotein TAG-72, the SK-1 antigen, tumor-associated epitopes that are present in elevated levels in the sera of patients with colon and/or pancreatic cancer, cancer-associated epitopes or proteins expressed on breast, colon, squamous cell, prostate, pancreatic, lung, and/or kidney cancer cells and/or on melanoma, glioma, or neuroblastoma cells, the necrotic core of a tumor, integrin alpha 4 beta 7, the integrin VLA-4, B2 integrins, TRAIL receptors 1, 2, 3, and 4, RANK, RANK ligand, TNF-α, the adhesion molecule VAP-1, epithelial cell adhesion molecule (EpCAM), intercellular adhesion molecule-3 (ICAM-3), leukointegrin adhesin, the platelet glycoprotein gp IIb/IIIa, cardiac myosin heavy chain, parathyroid hormone, rNAPc2 (which is an inhibitor of factor VIIa-tissue factor), MHC I, carcinoembryonic antigen (CEA), alpha-fetoprotein (AFP), tumor necrosis factor (TNF), CTLA-4 (which is a cytotoxic T lymphocyte-associated antigen), Fc-γ-1 receptor, HLA-DR 10 beta, HLA-DR antigen, L-selectin, IFN-γ, Respiratory Syncitial Virus, human imillimolarunodeficiency virus (HIV), hepatitis B virus (HBV), *Streptococcus mutans*, and *Staphylococcus aureus*.

Preparations of various fusion proteins can also be prepared using the inventive methods. Examples of such fusion proteins include proteins expressed as a fusion with a portion of an immunoglobulin molecule, proteins expressed as fusion proteins with a zipper moiety, and novel polyfunctional proteins such as a fusion proteins of a cytokine and a growth factor (i.e., GM-CSF and IL-3, MGF and IL-3). WO 93/08207 and WO 96/40918 describe the preparation of various soluble oligomeric forms of a molecule referred to as CD40L, including an immunoglobulin fusion protein and a zipper fusion protein, respectively; the techniques discussed therein are applicable to other proteins. Any of the above molecules can be expressed as a fusion protein including but not limited to the extracellular domain of a cellular receptor molecule, an enzyme, a hormone, a cytokine, a portion of an immunoglobulin molecule, a zipper domain, and an epitope.

The preparation of recombinant protein can be a cell culture supernatant, cell extract, but is preferably a partially purified fraction from the same. By "partially purified" means that some fractionation procedure, or procedures, have been carried out, but that more polypeptide species (at least 10%) than the desired protein or protein conformation is present. One of the advantages of the methods of the invention is that the preparation of recombinant protein can be at a fairly high concentration. Preferred concentration ranges are 0.1 to 20 mg/ml, more preferably from 0.5 to 15 mg/ml, and still more preferably from 1 to 10 mg/ml.

The preparation of recombinant protein can be prepared initially by culturing recombinant host cells under culture conditions suitable to express the polypeptide. The polypeptide can also be expressed as a product of transgenic animals, e.g., as a component of the milk of transgenic cows, goats, pigs, or sheep which are characterized by somatic or germ cells containing a nucleotide sequence encoding the polypeptide. The resulting expressed polypeptide can then be purified, or partially purified, from such culture or component (e.g., from culture medium or cell extracts or bodily fluid) using known processes. Fractionation procedures can include but are not limited to one or more steps of filtration, centrifugation, precipitation, phase separation, affinity purification, gel filtration, ion exchange chromatography, hydrophobic interaction chromatography (HIC; using such resins as phenyl ether, butyl ether, or propyl ether), HPLC, or some combination of above.

For example, the purification of the polypeptide can include an affinity column containing agents which will bind to the polypeptide; one or more column steps over such affinity resins as concanavalin A-agarose, heparin-toyopearl® or Cibacrom blue 3GA Sepharose®; one or more steps involving elution; and/or immunoaffinity chromatography. The polypeptide can be expressed in a form that facilitates purification. For example, it may be expressed as a fusion polypeptide, such as those of maltose binding polypeptide (MBP), glutathione-S-transferase (GST) or thioredoxin (TRX). Kits for expression and purification of such fusion polypeptides are commercially available from New England BioLab (Beverly, Mass.), Pharmacia (Piscataway, N.J.) and InVitrogcn, respectively. The polypeptide can be tagged with an epitope and subsequently purified by using a specific antibody directed to such epitope. One such epitope (FLAG®) is commercially available from Kodak (New Haven, Conn.). It is also possible to utilize an affinity column comprising a polypeptide-binding polypeptide, such as a monoclonal antibody to the recombinant protein, to affinity-purify expressed polypeptides. Other types of affinity purification steps can be a Protein A or a Protein G column, which affinity agents bind to proteins that contain Fc domains. Polypeptides can be removed from an affinity column using conventional techniques, e.g., in a high salt elution buffer and then dialyzed into a lower salt buffer for use or by changing pH or other components depending on the affinity matrix utilized, or can be competitively removed using the naturally occurring substrate of the affinity moiety. In one embodiment of the invention illustrated below, the preparation of recombinant protein has been partially purified over a Protein A affinity column.

Some or all of the foregoing purification steps, in various combinations, can also be employed to prepare an appropriate preparation of a recombinant protein for use in the methods of the invention, and/or to further purify the recombinant polypeptide after contacting the preparation of the recombinant protein with a reduction/oxidation coupling reagent. The polypeptide that is substantially free of other mammalian polypeptides is defined as an "isolated polypeptide".

The polypeptide can also be produced by known conventional chemical synthesis. Methods for constructing polypeptides by synthetic means are known to those skilled in the art. The synthetically-constructed polypeptide sequences can be glycosylated in vitro.

The desired degree of final purity depends on the intended use of the polypeptide. A relatively high degree of purity is desired when the polypeptide is to be administered in vivo, for example. In such a case, the polypeptides are purified such that no polypeptide bands corresponding to other polypeptides are detectable upon analysis by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). It will be recognized by one skilled in the pertinent field that multiple bands corresponding to the polypeptide can be visualized by

SDS-PAGE, due to differential glycosylation, differential post-translational processing, and the like. Most preferably, the polypeptide of the invention is purified to substantial homogeneity, as indicated by a single polypeptide band upon analysis by SDS-PAGE. The polypeptide band can be visualized by silver staining, Coomassie blue staining, and/or (if the polypeptide is radiolabeled) by autoradiography.

By "contacting" is meant subjecting to, and/or exposing to, in solution. The protein or polypeptide can be contacted while also bound to a solid support (e.g., an affinity column or a chromatography matrix). Preferably, the solution is buffered. In order to maximize the yield of protein with a desired conformation, the pH of the solution is chosen to protect the stability of the protein and to be optimal for disulfide exchange. In the practice of the invention, the pH of the solution is preferably not strongly acidic. Thus, preferred pH ranges are greater than pH 5, preferably about pH 6 to about pH 11, more preferably from about pH 7 to about pH 10, and still more preferably from about pH 7.6 to about pH 9.6. In one non-limiting embodiment of the invention using TNFR:Fc that is illustrated below, the optimal pH was found to be about pH 8.6. However, the optimal pH for a particular embodiment of the invention can be easily determined experimentally by those skilled in the art.

The reduction/oxidation coupling reagent is a source of reducing agents. Preferred reducing agents are free thiols. The reduction/oxidation coupling reagent is preferably comprised of a compound from the group consisting of reduced and oxidized glutathione, dithiothreitol (DTT), 2-mercaptoethanol, dithionitrobenzoate, cysteine and cystine. For ease of use and economy, reduced glutathione and/or reduced cysteine can be used.

The reduction/oxidation coupling reagent is present at a concentration sufficient to increase the relative proportion of the desired conformation. The optimal concentration of the reduction/oxidation coupling reagent depends upon the concentration of protein and number of disulfide bonds in the protein. For example, it has been found using a protein (TNFR:Fc) with 29 disulfide bonds at a concentration of 2 mg/ml (approximately 14 microM protein or 400 microM disulfide), a reduction/oxidation coupling reagent with 2 mM reduced thiols worked well to increase the relative proportion of the desired conformation. This corresponds to a ratio of about 35 free thiols to 1 disulfide bond. However, it was also found that ratios from 20 to 400 free thiols per disulfide also worked. Of course, the amount of thiol used for a particular concentration can vary somewhat depending upon the reducing capacity of the thiol, and can be easily determined by one of skill in the art.

Thus, generally, the concentration of free thiols from the reduction/oxidation coupling reagent can be from about 0.05 mM to about 50 mM, more preferably about 0.1 mM to about 25 mM, and still more preferably about 0.2 mM to about 20 mM.

In addition, the reduction/oxidation coupling reagent can contain oxidized thiols at approximately higher, equal or lower concentrations as the reduced thiol component. For example, the reduction/oxidation coupling reagent can be a combination of reduced glutathione and oxidized glutathione. It has been found through actual working examples, that a ratio of reduced glutathione to oxidized glutathione of from about 1:1 to about 100:1 (reduced thiols:oxidized thiols) can function equally well. Alternatively in another embodiment, the reduction/oxidation coupling reagent can be cysteine or a combination of cysteine and cystine. Thus, when oxidized thiols are included in the initial reduction/oxidation coupling reagent, the ratio of reduced thiols to oxidized thiols can in a preferred embodiment be from about 1:10 to about 1000:1, more preferably about 1:1 to about 500:1, still more preferably about 5:1 to about 100:1, even more preferably about 10:1.

Contacting the preparation of recombinant protein with a reduction/oxidation coupling reagent is performed for a time sufficient to increase the relative proportion of the desired conformation. Any relative increase in proportion is desirable, but preferably at least 10% of the protein with an undesired conformation is converted to protein with the desired conformation. More preferably at least 20%, 30%, 40%, 50%, 60%, 70% and even 80% of the protein is converted from an undesired to a desired conformation. Typical yields that have been achieved with the methods of the invention range from 40 to 80%. If the contacting step is performed on a partially or highly purified preparation of recombinant protein, the contacting step can be performed for as short as about 1 hour to about 4 hours, and as long as about 6 hours to about 4 days. It has been found that a contacting step of about 4 to about 16 hours or about 18 hours works well. The contacting step can also take place during another step, such as on a solid phase or during filtering or any other step in purification.

The methods of the invention can be performed over a wide temperature range. For example, the methods of the invention have been successfully carried out at temperatures from about 4° C. to about 37° C., however the best results were achieved at lower temperatures. A typical temperature for contacting a partially or fully purified preparation of the recombinant protein is about 4° C. to about 25° C. (ambient), but can also be performed at lower temperatures and at higher temperature.

The preparation of recombinant protein can be contacted with the reduction/oxidation coupling reagent in various volumes as appropriate. For example, the methods of the invention have been carried out successfully at the analytical laboratory-scale (1–50 mL), preparative-scale (50 mL–10 L) and manufacturing-scale (10 L or more). Thus, the methods of the invention can be carried out on both small and large scale with reproducibility.

In preferred aspects, the contacting step is performed in the absence of significant amounts of chaotropic agents such as, for example, SDS, urea and guanidium HCl. Significant amounts of chaotropic agents are needed to observe perceptible unfolding. Generally, less than 1 M chaotrope is present, more preferably less than 0.5 M, still more preferably less than 0.1 M chaotrope. A solution is essentially free of chaotrope (e.g., SDS, urea and guanidium HCl) when no chaotrope has been purposely added to the solution, and only trace levels (e.g., less than 10 mM) may be present (e.g., from the vessel or as a cellular byproduct).

Disulfide exchange can be quenched in any way known to those of skill in the art. For example, the reduction/oxidation coupling reagent can be removed or its concentration reduced through a purification step, and/or it can be chemically inactivated by, e.g., acidifying the solution. Typically, when the reaction is quenched by acidification, the pH of the solution containing the reduction/oxidation coupling reagent will be brought down below pH 7. Preferably, the pH is brought to below pH 6. Generally, the pH is reduced to between about pH 2 and about pH 7.

Determining the conformation of a protein, and the relative proportions of a conformation of a protein in a mixture, can be done using any of a variety of analytical and/or qualitative techniques. If there is a difference in activity between the conformations of the protein, determining the relative proportion of a conformation in the mixture can be

done by way of an activity assay (e.g., binding to a ligand, enzymatic activity, biological activity, etc.). For example, in one of the non-limiting embodiments described below, at least two different conformations of TNFR:Fc can be resolved by using a solid-phase TNF binding assay. The assay, essentially as described for IL-1R (Slack, et al., 1993, J. Biol. Chem. 268:2513–2524), can differentiate between the relative proportions of various protein conformations by changes in ligand-receptor binding association, dissociation or inhibition constants generated. Alternatively the binding results can be expressed as activity units/mg of protein.

If the two conformations resolve differently during chromatography, electrophoresis, filtering or other purification technique, then the relative proportion of a conformation in the mixture can be determined using such purification techniques. For example, in the non-limiting embodiments described below, at least two different conformations of TNFR:Fc could be resolved by way of hydrophobic interaction chromatography. Further, since far-UV Circular Dichroism has been used to estimate secondary structure composition of proteins (Perczel et al., 1991, Protein Engrg. 4:669–679), such a technique can determine whether alternative conformations of a protein are present. Still another technique used to determine conformation is fluorescence spectroscopy which can be employed to ascertain complimentary differences in tertiary structure assignable to tryptophan and tyrosine fluorescence. Other techniques that can be used to determine differences in conformation and, hence, the relative proportions of a conformation, are on-line SEC to measure aggregation status, differential scanning calorimetry to measure melting transitions (Tm's) and component enthalpies, and chaotrope unfolding.

By the term "isolating" is meant physical separation of at least one component in a mixture away from other components in a mixture. Isolating components or particular conformations of a protein can be achieved using any purification method that tends to separate such components. Accordingly, one can perform one or more chromatography steps, including but not limited to HIC, hydroxyapatite chromatography, ion exchange chromatography, affinity, and SEC. Other purification methods are filtration (e.g., tangential flow filtration), electrophoretic techniques (e.g., electrophoresis, electroelution, isoelectric focusing), and phase separation (e.g., PEG-dextran phase separation), to name just a few. In addition, the fraction of the preparation of recombinant protein that contains the protein in the undesired conformation can be treated again in the methods of the invention, to further optimize the yields of protein with the desired conformation.

For example, after treatment, protein samples can be prepared for hydrophobic interaction chromatography (HIC) by the following method. An equal volume of 850 mM sodium citrate, 50 mM sodium phosphate, pH 6.5 is added to the treated sample, and allowed to equilibrate to room temperature. After filtering (e.g., using a 0.22 □m filter), HIC chromatography is performed on a Toyopearl® Butyl 650-M resin (Tosoh Biosep LLC, Montgomeryville, Pa.), at a flow rate of 150 cm/hr, and a mass load of 2.1 mg•mL resin$^{-1}$. The column is prequilibrated with 3 column volumes of 425 mM NaCitrate, 50 mM $PO_4$ pH 6.5, sample is loaded, and then washed through with 3 column volumes of 425 mM NaCitrate, 50 mM $PO_4$ pH 6.5. Elution can be performed with a gradient of 425 mM NaCitrate, 50 mM $PO_4$ pH 6.5 to O mM NaCitrate, 50 mM $PO_4$ pH 6.5 in a total of 5 column volumes. Fractions can be collected during the elution. The column can be stripped with 3 column volumes of water followed by 3 column volumes of 0.1M NaOH. Using the methods of the invention accordingly, one can thus obtain preparations of TNFR:Fc that contain more than 85%, more than 90%, and even more than 95% of the TNFR:Fc present in the preparation in the most active conformation (Fraction #2). Compositions, including pharmaceutical compositions, of TNFR:Fc containing such proportions of Fraction #2 are therefore also provided by the invention.

The invention also optionally encompasses further formulating the proteins. By the term "formulating" is meant that the proteins can be buffer exchanged, sterilized, bulk-packaged and/or packaged for a final user. For purposes of the invention, the term "sterile bulk form" means that a formulation is free, or essentially free, of microbial contamination (to such an extent as is acceptable for food and/or drug purposes), and is of defined composition and concentration. The term "sterile unit dose form" means a form that is appropriate for the customer and/or patient administration or consumption. Such compositions can comprise an effective amount of the protein, in combination with other components such as a physiologically acceptable diluent, carrier, and/or excipient. The term "pharmaceutically acceptable" means a non-toxic material that does not interfere with the effectiveness of the biological activity of the active ingredient(s). Formulations suitable for administration include aqueous and non-aqueous sterile injection solutions which may contain anti-oxidants, buffers, bacteriostats and solutes which render the formulation isotonic with the blood of the recipient; and aqueous and non-aqueous sterile suspensions which may include suspending agents or thickening agents. In addition, sterile bulk forms and sterile unit forms may contain a small concentration (approximately 1 microM to approximately 10 mM) of a reduction/oxidation coupling reagent (e.g., glutathione, cysteine, etc.). The polypeptides can be formulated according to known methods used to prepare pharmaceutically useful compositions. They can be combined in admixture, either as the sole active material or with other known active materials suitable for a given indication, with pharmaceutically acceptable diluents (e.g., saline, Tris-HCl, acetate, and phosphate buffered solutions), preservatives (e.g., thimerosal, benzyl alcohol, parabens), emulsifiers, solubilizers, adjuvants and/or carriers. Suitable formulations for pharmaceutical compositions include those described in *Remington's Pharmaceutical Sciences,* 16th ed. 1980, Mack Publishing Company, Easton, Pa. In addition, such compositions can be complexed with polyethylene glycol (PEG), metal ions, and/or incorporated into polymeric compounds such as polyacetic acid, polyglycolic acid, hydrogels, dextran, etc., or incorporated into liposomes, microemulsions, micelles, unilamellar or multilamellar vesicles, erythrocyte ghosts or spheroblasts. Suitable lipids for liposomal formulation include, without limitation, monoglycerides, diglycerides, sulfatides, lysolecithin, phospholipids, saponin, bile acids, and the like. Preparation of such liposomal formulations is within the level of skill in the art, as disclosed, for example, in U.S. Pat. No. 4,235,871; U.S. Pat. No. 4,501,728; U.S. Pat. No. 4,837,028; and U.S. Pat. No. 4,737,323. Such compositions will influence the physical state, solubility, stability, rate of in vivo release, and rate of in vivo clearance, and are thus chosen according to the intended application, so that the characteristics of the carrier will depend on the selected route of administration. Sustained-release forms suitable for use include, but are not limited to, polypeptides that are encapsulated in a slowly-dissolving biocompatible polymer (such as the alginate microparticles described in U.S. Pat. No. 6,036,978), admixed with such a polymer (including

topically applied hydrogels), and or encased in a biocompatible semi-permeable implant.

The invention having been described, the following examples are offered by way of illustration, and not limitation.

## EXAMPLE 1

### Biophysical Assessment of TNFR:Fc Fractions #2 and #3

The p75 TNFR:Fc elutes off a hydrophobic interaction column (HIC) as three distinct peaks termed Fraction #1, Fraction #2 and Fraction #3 (see FIG. **1**). Fraction #2 is the desired fraction. Fraction #3 was of particular interest since it can comprise from 20 to 60% of the sample and has been shown to exhibit low TNF binding activity and A375 bioactivity in comparison with Fraction #2. Therefore, in the interest of understanding the differences between these two fractions and ascertaining what factors contribute to the loss in activity for Fraction #3 as it pertains to structure and conformation, biophysical studies were carried out. In this example, we analyzed Fraction #2 and Fraction #3 using Circular Dichroism, Fluorescence, on-line SEC/UV/LS/RI, and differential scanning calorimetry (DSC).

Materials and Methods

Materials: The starting material was TNFR:Fc in TMS buffer (10 mM Tris, 4% mannitol, 1% sucrose). HIC eluted fractions of this material were isolated as Fractions #2 and #3 for experimental studies described below.

Circular Dichroisim: Studies were carried out in the near (250–340 nm) and far-UV (190–250 nm) regions. The near-UV studies were carried out to elucidate differences in tertiary structure while the far-UV studies were used to characterize differences in secondary structure.

The near-UV Circular Dichroism measurements were conducted in the TMS solutions with the following concentrations. Starting material was diluted to 6.25 mg/ml while the Fractions #2 and #3 were evaluated at their existing concentrations of 9.4 and 5.4 mg/ml, respectively. A Circular Dichroism cell with a path length of 0.1 cm was used and scans conducted from 340 to 250 nm.

The far-UV Circular Dichroism measurements were performed with the protein buffer exchanged into 10 mM sodium phosphate (pH 7.0) and subsequently evaluated using a 0.1 cm path length cell scanned from 250 to 190 nm. Secondary structure composition was evaluated using convex constraint analysis (CCA) (Perczel et al., 1991, Protein Engrg. 4:669–679).

Fluorescence Spectroscopy: Samples were examined after dilution to approximately 50 microgram/ml using two different excitation wavelengths. Tyrosine and tryptophan fluorescence was examined with an excitation of 270 nm while tryptophan fluorescence was exclusively evaluated using an excitation of 295 nm (Lakowicz, J. R. in "Principles of Fluorescence Spectroscopy", Plenum Press, 1983. New York, N.Y., 342–343). Fluorescence scans extended from 300 to 440 nm for 270 nm excitation and from 310 to 440 nm for 295 nm excitation. Four consecutive scans were signal averaged for each spectrum. Normalized data were reported to evaluate differences in frequency arising from the samples.

Online-SEC/UV/LS/RI: The molecular weights of eluting components using size exclusion chromatography were ascertained using ultraviolet (UV @ 280 nm), light scattering (90°), and refractive index (RI) detection in series. This method has been well documented (see Arakawa et al., 1992, Anal. Biochem. 203:53–57 and Wen et al., 1996, Anal. Biochem. 240:155–166), and has an advantage of measuring the nonglycosylated molecular weights of proteins and peptides that are glycosylated. The SEC and UV data were collected using an Integral HPLC system (PerSeptive Biosystems, Inc.) with a BioSil-400-5 column (from BioRad) using a flow rate of 1 ml/min. The elution buffer consisted of 100 mM phosphate (pH 6.8) and 100 mM NaCl. A DAWN DSP multi-angle light scattering detector and Optilab DSP refractometer were both purchased from Wyatt Technology, Inc. Calibration standards to determine instrumental constants included BSA dimer, BSA monomer and ovalbumin (FIG. **2**).

Differential Scanning Calorimetry (DSC): Physical properties of unfolding were measured using a MicroCal MC-2 DSC instrument in upscan mode. Samples were prepared by buffer exchanging into the same TMS buffer at pH 7.4. Samples contained about 4 mg/ml protein and were evaluated against the buffer (absent protein) as a reference. The scan rate was 67° C./hr spanning the temperature regime from 20° C. to 90° C. Collected scans were subsequently converted into concentration normalized scans to better compare enthalpic behavior of unfolding transitions while taking into account differences in concentration (data reported as kcal/mole).

Results

Circular Dicliroism. The near-UV Circular Dichroism measurements expressed in terms of mean residue ellipticity are shown in FIG. **2**. Changes in a broad feature near 270 nm were evident between Fraction #2 and #3 as shown by a greater proportion of negative ellipticity in the spectrum of Fraction #3 (indicated by the arrow in FIG. **2**A). It was noted that the spectral behavior of the starting material closely matches that of Fraction #2 but does exhibit a subtle negative displacement in the same region surrounding 270 nm. This result seemed consistent as Fraction #3 made up a small part of the starting material and so its contribution to the overall ellipticity in this region was greatly reduced but in the same displacement direction. Reproducibility of the Fraction #3 spectrum confirmed the observed displacement of this sample to be real. With this in mind, and knowing that disulfides give rise to a broad negative elliptical feature in this region of the Circular Dichroism spectrum (see Kahn, P. C., 1978, Methods Enzymol. 61:339–378 and Kosen et al., 1981, Biochemistry 20:5744–5754), the near-UV Circular Dichroism spectrum was curve-fitted to estimate what the observed changes in this region mean in terms of tertiary structure. The results of the curve-fitted data are presented in FIG. **2**B and showed a small red-shift (3 nm) and enhanced negative displacement consistent with the contribution arising from a change in tertiary structure involving disulfides when comparing Fraction #3 with #2.

The far-UV Circular Dichroism has been used to estimate secondary structure composition of proteins (Perczel et al., 1991, Protein Engrg. 4:669–679). Secondary structure assignments using CCA were performed. Calculated spectra comprised of the sum of the secondary structure elements were compared with experimentally observed spectra and exhibited a good fit. The secondary structures of both fractions were comparable within limits of experimental precision (within 10%). Therefore, this experiment did not distinguish any differences regarding secondary structure for either of these two fractions.

Fluorescence Spectroscopy. Knowing that there were significant differences observed in the near-UV Circular

Dichroism region, fluorescence spectroscopy was employed to ascertain complimentary differences in tertiary structure assignable to tryptophan and tyrosine fluorescence. Using two excitation wavelengths, it was possible to determine that the spectra for all three cases considered (SM, Fraction #2 and #3) were super-imposable with fluorescence maxima near 338 nm. Since the three-dimensional structure of a given protein is responsible for emission maxima of native proteins, these results suggested that the average structure involving the intrinsic fluorophores, tryptophan and tyrosine was unperturbed.

On-line SEC/UV/LS/RI. The light scattering studies performed on-line with SEC yielded molecular weights of the main elution peak that were in agreement with the non-glycosylated polypeptide molecular weight of TNFR:Fc (e.g., 102 kD). Although there were clear differences in the compositions of eluting species evaluated with this technique, when comparing the elution profile of Fraction #3 with Fraction #2 (FIG. **3**A and B), the main peak comprising the majority component was measured to be 102.5±1.6 kD (Retention Volume=8.4 mL) and 101.9±2.1 kD (Retention Volume=8.3 mL), respectively. The precision was expressed as the standard deviation of 23 slices through the elution peak bracketed by the vertical dashed lines in FIG. **3**. It was also noted that a respectable signal of the descending shoulder for Fraction #3 permitted determination of the polypeptide molecular weight to be 78.1±3.7 kD (this evaluation considered 8 slices surrounding the peak labeled at 8.85 mL). As exhibited by the precision associated with the molecular weight determination of this component, this peak exhibited greater heterogeneity and as a result was suspect of greater polydispersion than the main peak. Fraction #3 also contained a significant amount of high molecular weight species consistent with the elution volume of a predominantly dimeric form of TNFR:Fc (near 7.5). Hence, it was determined that Fraction #3 is comprised of several species including aggregates and fragmented portions of the molecule.

Differential Scanning Calorimetry. DSC measurements carried out on the two fractions yielded significant differences in the unfolding of the TNFR moiety of the TNFR:Fc molecule (FIG. **4**). As shown more clearly in the baseline corrected data (FIG. **4**B), there is a 2.8° C. shift to lower temperature in the melting transition (Tm) when comparing a Tm of 52.5° C. (Fraction #2) with a Tm of 49.7° C. (Fraction #3). The transition is slightly broader for Fraction #3 with a half-width at half the transition maximum of 8° C. in comparison with Fraction #2 having a half-width of 6.5° C. This low temperature transition has been identified from thermal unfolding experiments of TNFR:Fc monomer to be due the TNFR domain of the molecule. Thermal transitions at 69.1° C. and 83.4° C. have been assigned to the Fc portion of the molecule. These latter two unfolding transitions align well and are comparable in terms of Tm's and component enthalpies.

Discussion

Among the methods tested, differences were observed in the near-UV Circular Dichroism and DSC measurements. Differential scanning calorimetry data support a loosening of structure that is assignable to the receptor moiety of the molecule with little change observed in the region of the Fc. The near-UV Circular Dichroism results suggested that disulfides are involved with tertiary structural changes associated with Fraction #3. These changes may arise as a consequence of buried disulfides gaining more exposure to the solvent and account for an increase in hydrophobicity as suggested by the small increase in retention time observed in the HIC elution of Fraction #3. It is interesting that there are no discernible differences found in the fluorescence data that would indicate such a change in conformational structure. If one considers the primary structure of TNFR:Fc in terms of the distribution of tyrosines (Y) and tryptophans (W), it becomes apparent that the region extending from the C-terminal portion of residue 104 of the TNFR domain to residue 296 of the N-terminal portion of the Fc (comprising 40% of the linear sequence of TNFR:Fc) is devoid of these intrinsic fluorophores. Therefore, one possible explanation consistent with the data might be that tertiary structure remote from the Fc hinge region is relatively unchanged while that from about residue C115 to C281 may be somewhat altered conformationally. This region of the molecule comprises 10 possible cysteines that may be affected with supposedly little consequence of structural change affecting local structure of tyrosines and tryptophans. It is noted that it is currently unknown as to how this molecule is folded and it would seem plausible that the cysteines that make up disulfides that are more remote from any given tryptophan or tyrosine residue would be logical suspects for tertiary structural changes that produce the observed near-UV Circular Dichroism results but exhibit little impact on the vicinal structure involving tyrosines and tryptophans. This idea does not preclude the possibility that there is some unusual change in structure within one or both of the TNFR arms that does not invoke an appreciable change in the net effect of fluorescence arising from tyrosines and tryptophans. The fact that the fluorescence data (which is insensitive to disulfides) show no change and the near-UV (that is sensitive to disulfides, tyrosines, and tryptophans) exhibits a small negative displacement consistent with disulfide structural modification does imply that disulfides play a role in the difference between Fractions #2 and #3.

In summarizing the remaining data generated concerning Fraction #3, aspects related to molecular weight and secondary structure were found to be indistinguishable from Fraction #2.

## EXAMPLE 2

### Disulfide Exchange Experiments on TNFR:Fc Fraction #3 with Glutathione

This experiment was designed to assess a variety of treatments to drive TNRF:Fc Fraction #3 into the conformation of Fraction #2 in a process amenable to large-scale production runs.

Materials and Methods

Materials. The starting material was TNFR:Fc as a Protein A elute, a pure HIC elute of Fraction #3, and a 50:50 mixture of HIC elutes Fraction #2 and Fraction #3. Buffers were 0.1 M citrate or 0.1 M Tris/glycine at pH 7.6, pH 8.6 or pH 9.6. Protein concentration of the TNFR:Fc was from 0.2 to 4.5 mg/mL. A redox coupling system of reduced glutathione and glutathione (GSH/GSSG at a ratio of 10:1) was added at 0.1 to 5 mM GSH. Incubation temperature was varied at 4 degrees, 22 degrees or 31 degrees Centigrade.

Methods. Disulfide exchange was quenched by acidification of the sample to pH 6 with 1 M acetic acid. Treated preparations of recombinant protein were characterized by analytical HIC, SEC (retention time, aggregate concentration) and solid-phase TNF binding assay to determine the percentage and yield of Fraction #2.

Results and Discussion

Treatment efficiency as a function of pH and GSH concentration. Significant % of the protein in Fraction #3 (at least 10%) was converted Fraction #2 when treatment was performed at both 0.1 mM GSH/pH 7.6 and 0.1 mM GSH/pH 8.6. However, efficiency was greatly improved (from 45% to almost 70%) when treatment was performed at 0.1 mM GSH/pH 9.6; 1 mM GSH/pH 7.6; 1 mM GSH/pH 8.6; and 1 mM GSH/pH 9.6. Thus, although treatment efficiency is sensitive to pH and free thiol concentration, it can be effectively performed over a wide range of these variables.

Temperature effects. Fraction #3 was treated at three different temperatures, 4° C., 22° C. and 31° C. The GSH concentration was held at 1 mM, and pH 8.6. After 16 hours, the treatment groups all exhibited significant conversion of Fraction #3 into Fraction #2, but conversion seemed slightly more efficient at the two lower temperatures.

Clone effects. Six different cell line clones, all producing TNFR:Fc, were tested in a standardized protocol based upon the above results. Specifically, a Protein A elution containing 0.4 to 0.7 mg/mL of TNFR:Fc (at about pH 4) was adjusted to pH 8.6 using 1M Tris/glycine (final concentration 0.1 M Tris/glycine). These solutions were adjusted to 1 mM EDTA and 2.5 mM GSH/0.25 mM GSSG and incubated at room temperature for about 16 hours. Disulfide exchange was quenched by acidification as described above.

Each of six different clones all showed improvement in production and yield of Fraction #2. The reduction of HIC Fraction #3 by treatment in the various clones was 64%, 72%, 77%, 78%, 78% and 83%. The increase in HIC Fraction #2 in the same clones was 37%, 64%, 78%, 70%, 44% and 54%, respectively. Percent increase in HIC Fraction #2 was well correlated with the % increase in Binding Units, as shown in FIG. **5**. Thus, the methods appeared generally applicable across all clones tested.

Binding assays. Three different preparations of TNFR:Fc were assayed in a solid-phase TNF binding assay. Samples 11-6 and 12 were eluants from a Protein A column. Sample 8085-47 was also eluted from a Protein A column, and then subjected to an additional HIC purification step; this sample contained exclusively Fraction #3. Samples were examined in the binding assay before and after disulfide exchange as described above. The results presented below in Table 1 show an increase in ligand binding activity after treatment of all samples with glutathione.

TABLE 1

TNF binding activity of TNFR:Fc before and after disulfide exchange

| Sample | Pre-exchange (activity/mg of protein) | Post-exchange (activity/mg of protein) | % Change |
|---|---|---|---|
| 11-6 | $4.16 \times 10^7$ | $5.73 \times 10^7$ | 27% |
| 12 | $4.36 \times 10^7$ | $6.13 \times 10^7$ | 29% |
| 8085-47 | $1.90 \times 10^7$ | $6.75 \times 10^7$ | 72% |

## EXAMPLE 3

### Disulfide Exchange Experiments on TNFR:Fc Treated with L-Cysteine

This experiment was designed to assess cysteine/cystine as reduction/oxidation coupling reagents for TNFR:Fc. The procedure allows assessment of change of HIC Fraction #3 into the conformation of Fraction #2 in a process amenable to large-scale production runs. The procedure can be performed on a purified Fraction #3, a mixture of Fractions #2 and #3, and/or following other separation techniques such as Protein A chromatography, with similar results.

Materials and Methods

The starting material was TNFR:Fc as a pure HIC elute of Fraction #3 or as a Protein A-eluted TNFR:Fc containing both Fraction #2 and #3. Buffers were 0.1 M citrate or 0.2 M Tris at pH 8.5. Protein concentration of the TNFR:Fc was 2.5 to 3 mg/mL.

A redox coupling system of L-cysteine (varying from 0 to 50 mM) was utilized. The procedure was also assessed +/−L-cystine (0.025 to 0.5 mM) and +/−1 mM EDTA. Incubation temperature was assessed at 4, 15, and 22 degrees Centigrade for 6, 18, and 48 hours. Disulfide exchange was quenched by acidification of the sample to pH 7 with $NaH_2PO_4$ or 0.85 M citrate. Treated preparations of recombinant protein were characterized by analytical HIC and SEC (retention time, aggregate concentration) to determine the percentage and yield of Fraction #2 and Fraction #3, cysteinylation and free sulfhydral assays.

Results and Discussion

Treatment efficiency as a function of L-cysteine concentration (0–5 mM). A significant percentage of the TNFR:Fc protein in HIC Fraction #3 (average 10%) was converted to Fraction #2 when treatment was performed with 0.25 mM L-cysteine in the absence of L-cystine or EDTA in four replicate samples (FIG. **6**). However, efficiency was greatly improved (from 45% to almost 70%) when treatment was performed at 1 mM L-cysteine or 5 mM L-cysteine (FIG. **6**). The effect of cystine in these reaction conditions varied with EDTA presence (see below). For a given cell culture batch (samples from four different cell culture batches were treated), the treatment process was reproducible.

Treatment efficiency as a function of higher L-cysteine concentration (5–50 mM). Higher concentrations of L-cysteine (5, 15 and 50 mM L-cysteine) used to treat TNFR:Fc resulted in a decrease in HIC Fraction #3 from the starting material in each case, but 5 mM L-cysteine was most effective in promoting the accumulation of Fraction #2 (FIG. **7**). It is estimated that higher concentrations of L-cysteine either significantly reduced the sulfhydryl moieties in the molecule or required too long to re-oxidize.

Treatment efficiency as a function of additional L-cysteine feeding. In order to attempt to increase disulfide exchange efficiency, TNFR:Fc was treated with 5 mM L-cysteine and incubated at 4 degrees Centigrade for 18 hours. Additional L-cysteine (0–5 mM) was then added, and the samples incubated at 4 degrees Centigrade for two additional days. Under these conditions, no significant effect on the ratio of HIC Fraction #3 to Fraction #2 was noted by additional L-cysteine feeding.

Effect of EDTA, cystine and L-cysteine. The effect of cystine (0–0.4mM) in combination with L-cystcine (5 mM) on TNFR:Fc was assessed in the presence or absence of 1 mM EDTA. Optimal results in the presence of 1 mM EDTA occurred with concentrations of cystine in the range of 0.1–0.2 mM.

Copper, temperature and time effects. TNFR:Fc was treated at with 5 mM L-cysteine at 4 degrees and 22 degrees Centigrade for either 6 or 18 hours. Completion of treatment of TNFR:Fc was assayed by copper addition followed by HIC. After 6 hours of incubation, disulfide exchange is more complete at 4 degrees than 22 degrees, and treatment is clearly more complete after 18 hours at 4 degrees Centigrade (FIGS. **8**A and **8**B).

Comparison of analytical- versus preparative-scale L-cysteine treatment efficiency. Based upon the treatment conditions optimized at small scale, TNFR:Fc (2.5 mg/mL in 0.2 M Tris, pH 8.5) in either 3 mL or 20 L quantities were treated with 5 mM L-cysteine (in the absence of cystine or EDTA), incubated at 4 degrees Centigrade for 18 hours, diluted with and equal volume of 850 mM sodium citrate, 50 mM sodium phosphate, pH 6.5 to quench the treatment, and chromatographed on HIC. Control samples of Preparative and Analytical scale TNFR:Fc had 63% and 68% Fraction #3, respectively. After treatment with the above conditions, Fraction #3 was reduced to 28% in both Preparative and Analytical scales. Therefore the treatment efficiency was 56% and 59% for the Preparative and Analytical samples, respectively (Table 2). This experiment demonstrates that the process is amenable to larger scale treatment.

TABLE 2

Analytical vs. Preparative Scale Disulfide Exchange Procedure

| | PREPARATIVE | | ANALYTICAL | |
|---|---|---|---|---|
| | Fraction #2 | Fraction #3 | Fraction #2 | Fraction #3 |
| Control | 37% | 63% | 32% | 68% |
| Exchange | 72% | 28% | 72% | 28% |
| Exchange "efficiency" | | 56% | | 59% |

Thus, although treatment redox efficiency is affected by free thiol concentration, temperature and time, it can be effectively optimized and performed over a wide range of variables. The treatment protocols can also be performed on both small and large scale with reproducibility.

The present invention is not to be limited in scope by the specific embodiments described herein, which are intended as single illustrations of individual aspects of the invention, and functionally equivalent methods and components are within the scope of the invention. Indeed, various modifications of the invention, in addition to those shown and described herein will become apparent to those skilled in the art from the foregoing description and accompanying drawings. Such modifications are intended to fall within the scope of the appended claims.

What is claimed is:

**1**. A method comprising:

contacting a preparation of a recombinant soluble form of a p75 TNF-receptor that has been produced by mammalian cells with a reduction/oxidation coupling reagent, at a pH of about 7 to about 11, and isolating a fraction of the preparation of the recombinant soluble form of the p75 TNF-receptor with a desired conformation, wherein the desired conformation has a higher binding affinity than an undesired conformation for a cognate ligand of the p75 TNF-receptor.

**2**. The method of claim **1** wherein the recombinant soluble form of the p75 TNF-receptor contains at least two domains.

**3**. The method of claim **2** wherein at least one domain of the recombinant soluble form of the p75 TNF-receptor has a stable conformation, and at least one domain of the protein has an unstable conformation.

**4**. The method of claim **1** wherein the recombinant soluble form of the p75 TNF-receptor is a Fc fusion protein.

**5**. The method of claim **4** wherein the preparation of the recombinant soluble form of the p75 TNF-receptor has been purified from a Protein A or Protein G column.

**6**. The method of claim **1** wherein the pH is from about 7 to about 10.

**7**. The method of claim **6** wherein the pH is about 7.6 to about 9.6.

**8**. The method of claim **7**, wherein the pH is about 8.6.

**9**. The method of claim **1** wherein the reduction/oxidation coupling reagent comprises glutathione.

**10**. The method of claim **9** wherein the ratio of reduced glutathione to oxidized glutathione is about 1:1 to about 100:1.

**11**. The method of claim **1** wherein the reduction/oxidation coupling reagent comprises cysteine.

**12**. The method of claim **1** wherein the contacting step is performed for about 4 to about 16 hours.

**13**. The method of claim **1** wherein the contacting step is performed at about 25° C.

**14**. The method of claim **1** wherein the contacting step is performed at about 4° C.

**15**. The method of claim **1** wherein the contacting step is quenched by acidification.

**16**. The method of claim **1** wherein the isolating step comprises one or more chromatography steps.

**17**. The method of claim **1** wherein the protein concentration of the recombinant soluble form of the p75 TNF-receptor is from about 0.5 to about 10 mg/ml.

**18**. The method of claim **1** wherein the ratio of reducing thiols in the reduction/oxidation coupling reagent to disulfide bonds in the protein is about 320:1 to about 64,000:1 (reducing thiols: disulfide bond).

**19**. The method of claim **1** further comprising formulating the fraction of the preparation of the recombinant soluble form of the p75 TNF-receptor with the desired conformation in a sterile bulk form.

**20**. The method of claim **1** further comprising formulating the fraction of the preparation of the recombinant soluble form of the p75 TNF-receptor with the desired conformation in a sterile unit dose form.

**21**. The method of claim **1** wherein the desired conformation has a higher binding affinity for a TNF.

**22**. The method of claim **21** wherein the TNF is TNF-alpha.

**23**. The method of claim **1** wherein the contacting step is performed in a solution essentially free of chaotrope.

**24**. A method of promoting a desired conformation of a glycosylated recombinant soluble form of a p75 TNF-receptor, the method comprising

contacting a preparation of the glycosylated recombinant soluble form of the p75 TNF-receptor that contains a mixture of at least two configurational isomers of the glycosylated recombinant soluble form of the p75 TNF-receptor with a reduction/oxidation coupling reagent for a time sufficient to increase the relative proportion of the desired configurational isomer and

determining the relative proportion of the desired configurational isomer in the mixture,

wherein the desired configurational isomer has a higher binding affinity than an undesired configurational isomer for a cognate ligand of the p75 TNF-receptor.

**25**. The method of claim **24** wherein the glycosylated recombinant soluble form of the p75 TNF-receptor contains at least two domains.

**26**. The method of claim **25** wherein at least one domain of the glycosylated recombinant soluble form of the p75 TNF-receptor has a stable conformation, and at least one domain of the glycosylated recombinant soluble form of the p75 TNF-receptor has an unstable conformation.

**27**. The method of claim **24** wherein the glycosylated recombinant soluble form of the p75 TNF-receptor is a Fc fusion protein.

**28**. The method of claim **27** wherein the preparation of the glycosylated recombinant soluble form of the p75 TNF-receptor has been purified from a Protein A or Protein G column.

**29**. The method of claim **24** wherein the pH is from about 7 to about 10.

**30**. The method of claim **29** wherein the pH is about 8.6.

**31**. The method of claim **24** wherein the reduction/oxidation coupling reagent is selected from the group consisting of glutathione, cysteine, DTT (dithiothreitol), 2-mercaptoethanol and dithionitrobenzoate.

**32**. The method of claim **31** wherein the reduction/oxidation coupling reagent comprises reduced glutathione.

**33**. The method of claim **32** wherein the reduced glutathione is at a concentration of about 1 mM to about 10 mM.

**34**. The method of claim **31** wherein the reduction/oxidation coupling reagent comprises reduced cysteine.

**35**. The method of claim **31** wherein the ratio of reducing thiols in the reduction/oxidation coupling reagent to disulfide bonds in the protein is about 320:1 to about 64,000:1 (reducing thiols: disulfide bond).

**36**. The method of claim **24** wherein the protein concentration is from about 0.5 to about 10 mg/ml.

**37**. The method of claim **24** wherein the contacting step is performed for about 4 to about 16 hours.

**38**. The method of claim **24** wherein the contacting step is performed at about 25° C.

**39**. The method of claim **24** wherein the contacting step is performed at about 4° C.

**40**. The method of claim **24** wherein the contacting step is quenched by acidification.

**41**. The method of claim **24** wherein the determining step comprises one or more chromatography steps.

**42**. The method of claim **24** wherein the determining step comprises a binding reaction.

**43**. The method of claim **24** comprising isolating a fraction of the preparation of the glycosylated recombinant soluble fonn of the p75 TNF-receptor with the desired configurational isomer.

**44**. The method of claim **43** comprising formulating the desired configurational isomer in a sterile unit dose form.

**45**. The method of claim **24** wherein the desired configurational isomer has a higher binding affinity for a TNF.

**46**. The method of claim **45** wherein the TNF is TNF-alpha.

**47**. The method of claim **24** wherein the contacting step is performed in a solution essentially free of chaotrope.

**48**. A method comprising formulating into sterile unit dose form a recombinant soluble form of the p75 TNF-receptor that has been produced by mammalian cells, contacted with a reduction/oxidation coupling reagent, and isolated from the fraction of the protein with an undesired conformation, wherein the undesired conformation has a lower binding affinity for a cognate ligand of the p75 TNF-receptor.

**49**. The method of claim **48** wherein the contacting step has been performed in a solution essentially free of chaotrope.

* * * * *